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Characterisation of livestock-associated methicillin-resistant *Staphylococcus aureus* strains isolated in Hungary

Ph.D. thesis

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Abbreviations

AST: antimicrobial susceptibility testing

bp: base pair

CLSI: Clinical Laboratory Standard Institute

cgMLST: core genome multilocus sequence typing

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

ECOFF: epidemiological cut-off value

EFSA: European Food Safety Authority

EHA-MRSA: equine hospital-associated methicillin-resistant *Staphylococcus aureus*

EUCAST: European Committee on Antibiotic Susceptibility Testing

gyrA gene: gene of DNA gyrase, A subunit

LukPQ and *lukPQ*: equine-specific bicomponent leukocidin and its gene

MIC: minimum inhibitory concentration

MLST: multilocus sequence typing

MGE: mobile genetic element

MLVA: multi-locus variable number of tandem repeats analysis

MRSA: methicillin-resistant *Staphylococcus aureus*

PCR: polymerase chain reaction

PFGE: pulsed-field gel electrophoresis

$PhLOPS_A$: phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics

PVL: Panton-Valentine leukocidin

SCC_{mec}: *Staphylococcus* chromosome cassette *mec* element

SNP: single nucleotide polymorphism

spa: *Staphylococcus* protein A gene

SSTI: skin and soft tissue infections

ST: sequence type

Ta: annealing temperature

Tm: melting temperature

UPGMA: unweighted pair group method with arithmetic mean

wgMLST: whole genome multilocus sequence typing

1. Summary

Staphylococcus aureus is a worldwide facultative pathogenic bacterium, which may be carried asymptomatically by 10–30% of the human and domesticated mammalian populations. The *S. aureus*-related syndromes range from benign skin and soft tissue infections to life-threatening necrotic pneumonia, endocarditis, and osteomyelitis. By the extensive use of antibiotics, the methicillin-resistant variants of *S. aureus* (MRSA) nowadays are among the most successful and best known multidrug-resistant pathogens. Strains of MRSA have first emerged in human healthcare settings and later have also been observed in the community without any link to healthcare. More recently, MRSA spreads rapidly in several species of domestic animals. Thus, the livestock-associated (LA-) MRSA belonging to the clonal complex (CC) 398 has become the most abundant lineage in Europe. This clonal lineage was found to be prevalent in pig herds in some European countries in the early 2000s and associated isolates were also recovered from diseased farm workers. Later, this lineage was proved to be of human origin, which retained its ability to successfully colonise and infect the human host.

According to the European Food Safety Authority baseline study, in 2008, Hungary reported 3 positive farms out of the investigated 181 holdings. However, no such further official estimation of MRSA in pig settings has been made in the country since then. The sampling of 40 pig farms, in 2019, showed a dramatic increase in the occurrence of MRSA in the Hungarian swine industry, as 33 (83%) of them were tested positive. The key role of purchase networks – both domestic and transboundary ones– was reinforced in the spread of the pathogen, by the comparison of whole genome sequences, including other European isolates. Also, a high prevalence (70%) was observed in the investigated swine professionals (veterinarians and farm managers) working on these farms. The isolated LA-MRSA strains belonged exclusively to the livestock-associated clonal complex 398, and the WGS analyses revealed close relatedness between most farm isolates and isolates from swine professionals and diseased patients, pointing to the spill-over of the LA-MRSA to the human host. Besides their high zoonotic potential, the antibiotic resistance of these strains is also of concern. Half of the strains, isolated from pig farms, showed resistance or a non-wild-type phenotype to at least six tested antibiotics (besides beta-lactams), and along with human isolates, they carried eight different types of multidrug-resistance genes, including *cfr*, which encodes resistance to five classes of antimicrobials.

In dairy cattle *S. aureus* is considered one of the most important bacteria causing contagious clinical or subclinical intramammary infection. However, the mastitis of MRSA origin, is an occasional observation and rarely causes a stock-level problem. The last domestic prevalence estimates on MRSA of dairy origin were made in the early 2000s and resulted in as low as

0-0.6%. In the frame of a passive surveillance, 626 individual *S. aureus* strains isolated from cow milk samples of 42 farms were collected and characterized between July 2017 and December 2018. The surveillance resulted in four (0.5%) MRSA CC398 strains from three (7.1%) farms, suggesting an unchanged occurrence and significance of MRSA of dairy origin in Hungary during the past decade. A retrospective investigation of the Veterinary Diagnostic Directorate's culture collection (Budapest, Hungary) revealed 27 MRSA strains originating from ten dairy farms. The isolates belonged to various sequence types (STs) and clonal complexes (ST1, ST22, CC97, and ST398), of which all can colonize and infect the human host. Most investigated isolates were resistant to three or more antimicrobial classes, hence the low host specificity and multidrug-resistance of the strains calls for periodic revision on the role and distribution of the MRSA in the Hungarian dairy sector.

MRSA infection in horses means a serious challenge to veterinary medicine, especially in equine healthcare settings, where the pathogen can become a resident nosocomial pathogen. The infections can result in life-threatening conditions, and even the benign cases may increase the treatment costs and prolong the time needed for full recovery. Equine MRSA was first recognized in the Department and Clinic of Equine Medicine (DCEM) of the University of Veterinary Medicine Budapest (Budapest, Hungary) in 2011.

Equine bacterial isolates have been collected and deposited in the culture collection of the Diagnostic Laboratory since 2009. By the molecular investigation of this collection series of outbreaks were identified between 2010 and 2018, caused by two distinct lineages of the equine hospital-associated (EHA-) MRSA CC398–t011–SCC$_{mec}$IV, dubbed as lineage 1 (L1) and lineage 2 (L2). Strains of L1 were totally replaced by that of L2 in 2011, and the latter gradually gained additional resistance and virulence genes while residing in the hospital environment, most of them encoded on mobile genetic elements. Changes in the resistance pattern can be attributed to the introduction of the relevant agent into the hospital environment, and thus regarded as an evolutionary response to the selective pressure. The emergence of the equine specific leukocidin LukPQ within the clonal lineage, suggests a further step towards successful host adaptation. The same time-related pattern of microevolution was observed when MRSA strains from clinical specimens of other private veterinary practices, including a further equine clinic (Clinic 2), were analysed retrospectively from the timeframe of 2008-2018, suggesting a common origin of strains. Besides the genomic evidence, epidemiologic links could also be identified between the horses and equine clinics in some cases.

The observation of two apparently independent MRSA outbreaks in 2018, involving Clinic 2 and three related farms, and another in 2019 observed in a stud, resulted in the isolation of the same genetic lineage L2. The isolates showed only slight differences in their drug resistance

patterns, however, the changes suggest local adaptive microevolutionary steps. Apart from these outbreaks, the pathogen is apparently rare in the horse population outside the clinic, as none of the 325 horses from 24 farms tested positive, and only 6% of 128 horses admitted to the clinic carried MRSA strains. Of these, all but one belonged to L2, supporting the clonal uniformity of the Hungarian equine-derived MRSA strains further.

The caretakers and the environment of horses can act as a potent reservoir of EHA-MRSA. In both clinics more than 25% of the staff carried the pathogen, while in each affected farm the positivity level was over 20% among the workers. MRSA could be isolated in the environment of the horses in all investigated cases. The role of heavy contamination of the environment was underlined by the large proportion (47%) of MRSA positive horses leaving the DCEM.

The whole genome-based comparison of isolates to other European data reinforced the very close relatedness of the Hungarian L2 isolates. The results indicate a gradual adaptive micro-evolution of the clonal lineage taking place presumably in only a few epicentres, as well as its occasional spread from the epicentres into the horse population in Hungary. Such epicentres may include, besides the equine clinics, certain intensive horse-keeping establishments, e.g. studs, where the conditions required for the enrichment of the pathogen are present. Future control programs should therefore focus on these settings.

Összefoglalás

A *Staphylococcus aureus* világszerte elterjedt fakultatív patogén baktérium, amelyet az emberi és háziasított emlőspopulációk 10-30%-a tünetmentesen hordozhat. A *S. aureus*-szal kapcsolatos kórképek a jóindulatú bőr- és lágyrészfertőzésektől az életveszélyes elhalásos tüdőgyulladásig, az endocarditisig és az osteomyelitisig terjednek. Az antibiotikumok széles körű alkalmazásával a *S. aureus* (MRSA) meticillin rezisztens változatai napjainkban a legsikeresebb és legismertebb multirezisztens kórokozók közé tartoznak. Az MRSA törzsei először a humán-egészségügyi intézményekben jelentek meg, később pedig olyan populációkban is megfigyelték őket, amelyeknek nem volt igazolható kapcsolatuk az egészségügyi ellátással. Az utóbbi időben az MRSA gyorsan terjed számos háziállatfajban is. Így az állattartáshoz köthető (livestock-associated, LA-) MRSA, amelynek törzsei jellemzően a 398-as klonális komplexhez tartoznak, Európa legelterjedtebb klonális vonalává vált. A 2000-es évek elején vált ismertté, hogy ez a klonális vonal Európa számos országának sertésállományaiban jelen van, és hasonló genotípusú törzseket izoláltak klinikai beteg mezőgazdasági dolgozók mintáiból is. Későbbi vizsgálatok igazolták, hogy ez a klonális vonal emberi eredetű, és megőrizte azt a képességét, hogy sikeresen kolonizálja és megfertőzze az emberi gazdaszervezetet.

Az Európai Élelmiszerbiztonsági Hatóság a témában végzett alapfelmérése szerint 2008-ban Magyarország 181 vizsgált sertéstelepe közül három volt MRSA-pozitív. Az országban azonban azóta nem készült hasonló hivatalos becslés az MRSA-val kapcsolatban a sertéstartásban. 2019-ben 40 sertéstelep mintázása igazolta, hogy drámai mértékben megnőtt az MRSA előfordulása a magyar sertéságazatban, a vizsgált telepek közül 33 (83%) pozitívnak bizonyult. A kórokozó elterjedésében a – mind a hazai, mind a határokon átnyúló – állatmozgások kulcsszerepét a hazai és más európai izolátumok teljesgenom-szekvenciáinak összehasonlítása erősítette meg. Szintén nagy előfordulási arány (70%) volt megfigyelhető az ezeken a telepeken dolgozó sertésegészségügyi és sertéstenyésztő szakemberek (állatorvosok és telepvezetők) körében. Az izolált LA-MRSA-törzsek kizárólag az állattenyésztéshez köthető 398-as klonális komplexhez tartoztak. A teljesgenom-elemzések szoros rokonságot mutattak ki a legtöbb telepi izolátum, valamint a sertéses szakemberekből és a humán klinikai mintákból származó izolátumok között, ami az LA-MRSA közegészségügyben való megjelenését igazolja. Amellett, hogy a törzsek zoonotikus potenciálja nagy, kiterjedt antibiotikumrezisztenciájuk is aggodalomra ad okot. A sertéstelepekről izolált törzsek fele legalább hat vizsgált antibiotikummal szemben mutatott rezisztenciát vagy nem vad fenotípust (a béta-laktám antibiotikumokon kívül). A sertéstelepi és humán klinikai izolátumokat vizsgálva, ezek nyolc különböző típusú multidrog-rezisztenciagént hordoztak, többek között a *cfr* gént, amely öt antimikrobiális hatóanyagcsoporttal szemben kódol rezisztenciát.

Tejelő szarvasmarháknál a *S. aureus* az egyik legfontosabb fertőző klinikai vagy szubklinikai tőgygyulladást okozó baktérium. Az MRSA okozta tőgygyulladás azonban csak alkalmi megfigyelés és ritkán jelent állományszintű problémát. A nyers tejből származó MRSA hazai előfordulására vonatkozó legutolsó vizsgálatok a 2000-es évek elején készültek, és mindössze 0–0,6% prevalenciát mutattak. Egy 2017 júliusa és 2018 decembere között végzett passzív surveillance keretében 42 gazdaság tehéntejmintáiból izolált 626 egyedi *S. aureus*-törzset gyűjtöttünk össze és jellemeztünk. A megfigyelés eredményeként három gazdaságból (7,1%) négy (0,5%) MRSA CC398 törzset azonosítottunk. A Nébih Állategészségügyi Diagnosztikai Igazgatóság (Budapest) törzsgyűjteményének retrospektív vizsgálatával 27 MRSA-törzset azonosítottunk, amelyek tíz tejgazdaságból származnak. Az izolátumok különböző szekvenciatípusokhoz (ST-k), illetve klonális komplexekhez tartoztak (ST1, ST22, CC97 és ST398). Ezek mindegyike képes kolonizálni és megfertőzni az emberi szervezetet. A legtöbb vizsgált izolátum három vagy több antimikrobiális hatóanyagcsoportra rezisztensnek bizonyult. Ezért a törzsek kismértékű gazdaspecificitása, valamint multidrog-rezisztenciája megkívánja az MRSA szerepének és elterjedésének időszakos felülvizsgálatát a magyar tejágazatban.

A lovak MRSA-fertőzése komoly kihívást jelent az állatgyógyászat számára, különösen a lovak egészségügyi ellátásában, ahol a baktérium tartósan nozokomiális kórokozóvá válhat. A fertőzések életveszélyes kórképeket eredményezhetnek; sőt, a jóindulatú esetek is megnövelhetik a kezelési költségeket és meghosszabbíthatják a kezelt lovak teljes felépüléséhez szükséges időt. Lóeredetű MRSA-t először 2011-ben azonosítottak az Állatorvostudományi Egyetem (Budapest) Lógyógyászati Tanszékén és Klinikáján (LTK).

A lovakból izolált baktériumokat 2009 óta gyűjtik és helyezik el a Diagnosztikai Laboratórium törzsgyűjteményében. Ennek a gyűjteménynek a molekuláris vizsgálatával 2010 és 2018 között olyan járványmeneteket azonosítottunk, amelyeket a lókórházhoz köthető (equine hospital associated; EHA-) CC398–t011–SCC$_{mec}$IV genotípusú MRSA két különböző klonális vonala okozott (L1 és L2 néven jelölve). Az L1 törzseket 2011-ben teljesen felváltották az L2 törzsek, és ez utóbbi törzsei fokozatosan további olyan rezisztencia- és virulenciagénekre tettek szert a kórházi környezetben, amelyek többségükben mobilis genetikai elemeken kódoltak. A rezisztencia mintázatában bekövetkezett változások az adott hatóanyagok kórházi használatához köthetők, ezért a szelektív nyomásra adott evolúciós válasznak tekinthetők. A lóspecifikus leukocidin (LukPQ) megjelenése a klonális vonalon belül egy további lépés a sikeres gazdaadaptáció felé. Ugyanezt az idővel összefüggő mikroevolúciós mintázatot figyeltük meg, amikor más magánállatorvosi praxisok klinikai mintáiból származó MRSA-törzseket, köztük egy további lóklinika (2. lóklinika) mintáit elemeztük retrospektív módon a 2008-2018 közötti időszakra vonatkozóan. Mindez a törzsek közös eredetére utal. A genomikai bizonyítékok mellett egyes esetekben epidemiológiai kapcsolatok is kimutathatók voltak a lovak és a lóklinikák között.

Két, az LTK-tól látszólag független MRSA-járványmenet megfigyelése ugyanazon L2 genetikai vonal izolálását eredményezte. A járványmenetek 2018-ban a 2. lóklinikán és három kapcsolódó lovardában, valamint 2019-ben egy ménesben zajlottak. Az izolátumok antibiotikum-rezisztenciájában csak csekély eltérések mutatkoztak, azonban a megfigyelt különbségek lokális adaptív mikroevolúciós lépésekre utalnak. Ezektől a járványoktól eltekintve a kórokozó vélhetően ritka a klinikán kívüli lópopulációban, ugyanis 24 lovardában tartott 325 lóból nem sikerült MRSA-t izolálnunk, míg az LTK-ra felvett 128 lónak is csak 6%-a hordozott MRSA-törzset. Ezek közül egy kivételével mindegyik az L2 vonalhoz tartozott, ami tovább erősíti a magyar lovakból származó MRSA-törzsek klonális leszármazását.

A lovak gondozói, valamint az állatok környezete az EHA-MRSA jelentős forrása lehet. Mindkét klinikán a dolgozók több mint negyede hordozta a kórokozót, míg mindegyik érintett lótartó helyen 20% feletti volt a pozitivitás a dolgozók körében. Az MRSA minden vizsgált esetben izolálható volt a lovak környezetéből is. A környezet erős szennyeződésének szerepét tovább hangsúlyozta az LTK-t elhagyó MRSA-pozitív lovak nagy aránya (47%).

Az izolátumok teljesgenom-alapú összehasonlítása más európai adatokkal megerősítette a magyar L2 izolátumok nagyon szoros rokonságát. Az eredmények a klonális leszármazási vonal feltehetően csak néhány epicentrumban lezajló, fokozatos adaptív mikroevolúcióját, valamint az epicentrumokból a magyarországi lóállományba történő esetenkénti átterjedését jelzik. Ilyen epicentrumok lehetnek a lóklinikán kívül bizonyos intenzív lótartó helyek, pl. ménesek, ahol a kórokozó feldúsulásához szükséges feltételek adottak. A jövőbeli kontrollprogramoknak ezért ezekre a létesítményekre kell összpontosítaniuk.

2. Introduction and literature overview

By now, methicillin-resistant *Staphylococcus aureus* (MRSA) has become one of the most successful and best known multidrug-resistant pathogens, posing a serious challenge to both public and animal health (Cuny et al., 2015c). The European Centre for Disease Prevention and Control (ECDC) considers the surveillance of multidrug-resistant pathogens a high-priority task. According to its most recent data, among the *Staphylococcus aureus* strains isolated from human samples in the Southern and Eastern European countries the proportion of invasive MRSA strains may exceed 50% (EFSA, 2010). According to the report issued by the National Centre for Epidemiology in 2016, in the period between 2005 and 2014 MRSA was the most common nosocomial multidrug-resistant bacterium in Hungary, with an annual incidence of 3.5–6 infections per 100,000 hospital days (Országos Epidemiológiai Központ, 2016).

Many of the MRSA genotypes occur in both humans and animals, enabling cross-infections among species and a further increase of the fitness of MRSA. The remarkable adaptability of the bacterium and, within that, its rapid evolutionary responses to different antimicrobials call for the soonest possible exploration of the relevant epidemiological situation in Hungary and a detailed characterisation of the isolated strains. This workflow attempted to contribute to this task by investigating the occurrence and genetic characteristics of livestock-associated MRSA strains isolated from certain large animal species, humans, and environmental samples.

Staphylococcus aureus and methicillin-resistant *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a Gram-positive, facultative anaerobic bacterium belonging to the phylum Firmicutes. The microorganism is characterised by nonmotile, non-spore-forming, spherical cells of 0.5 to 1.5 µm in diameter, occurring as single cocci or forming irregular clusters. They are among the most resistant non-spore forming bacteria and can grow in the presence of 10% NaCl between 18°C and 40°C. The species is named after its variants producing a yellow pigment. Regarding pathogenicity, it is a facultative pathogenic bacterium which may be carried asymptomatically by 10–30% of the adult human population (Gordon et al., 2008; Wertheim et al., 2005). This bacterium is one of the major pathogens in both human and veterinary medicine (Fitzgerald, 2012).

Semisynthetic methicillin is a member of the penicillinase-stable beta-lactam antibiotic family. Within a few years after its introduction in 1959, the appearance of methicillin-resistant *S. aureus* strains was described (Barber, 1961). That resistance was caused by the emergence of the enzyme protein variant PBP2' or PBP2a, which originally served as the target of beta-lactam antibiotics. The beta-lactam compounds cannot bind to this variant, which results in resistance to these antibiotics. The gene segment encoding PBP2a, *mecA* is embedded in a so-called staphylococcal cassette chromosome mec (SCC_{mec}) is a mobile genetic element (MGE) capable

of easy spread both within and between bacterium species. This mobility partly explains the wide occurrence of methicillin resistance within the *Staphylococcus* genus (Weterings et al., 2017). SCC_{mec} had probably originated from a coagulase-negative *Staphylococcus* species and became part of the genome of *S. aureus* only later. Eleven main types of SCC_{mec} have been identified in the species *S. aureus* so far, and several variants of these main types are known. Many of them encode not only the gene, responsible for beta-lactam resistance, but also some other antibacterial resistance genes (Shore et al., 2013).

Disease, pathogenicity, and virulence factors

Ten to thirty percent of the human and domesticated mammal population asymptomatically carry *S. aureus* on at least one body site, most frequently in the anterior nares, and with lesser extent on the skin or in the gastrointestinal tract. The risk of getting infected is higher among carrier individuals compared to non-carriers (Wertheim et al., 2005), however fomites, like medical devices and dust particles also play an important role, especially in the nosocomial transmission. Once present, the bacterium may access to tissues and bloodstream directly through the epithelium or open wounds by using different virulence mechanisms. It can also utilize indwelling medical devices, like catheters as entry site, where it forms a biofilm soon after insertion (Otto, 2018). The syndromes caused by *S. aureus* range from benign skin and soft tissue infections (SSTI) through *S. aureus* bacteraemia (SAB) to life-threatening necrotic pneumonia, endocarditis, and osteomyelitis (Gordon et al., 2008; Tong et al., 2015).

The matter of virulence is very complex and controversial in the case of *S. aureus*. Unlike many other pathogens, which often rely on one or a few virulence determinants, *S. aureus* has an armour of toxins, and a vast array of protein and non-protein factors that enable host colonization, immune-evasion, and direct killing of host immune cells in the course of infection (Cheung et al., 2021). Most virulence determinants are encoded in the core genome, hence considered ubiquitous among strains, like alpha-toxin, haemolysins, or the microbial surface components recognizing adhesive matrix molecules (MSCRAMM). Others are encoded in the accessory genome, on mobile genetic elements, like prophages and *Staphylococcus* pathogenicity islands (SaPIs). Such virulence factors are the bi-component leukocidins and immune-evasion factors, which may highly contribute to the niche-adaptation of the pathogen (Goerke et al., 2006; Spaan et al., 2017) The bi-component leucocidins play a key role in the elimination of host neutrophils. In most cases, the toxins' S subunit host-specifically recognizes the cell receptor of neutrophils and after the assembly of the other, F subunits, a membrane pore is formed. The human-associated Panton-Valentine leukocidin (PVL) enhances the virulence of strains and it seems to play a major role in the pathogenesis of severe pneumonia (Otto, 2013). The horse-specific leukocidin LukPQ is a recent discovery and similarly to PVL, it is encoded on prophages, called ϕSaeq1. Little is known about LukPQ's role under *in-vivo* circumstances, although its host-

specific neutrophil killing activity was already demonstrated *in-vitro* (Koop et al., 2017). LukMF' is associated with bovine isolates and shows a remarkable tropism towards bovine but not human phagocytes (Spaan et al., 2017).

Some further important human-associated staphylococcal toxins are encoded also on prophages, like the genes of the so-called immune evasion cluster (IEC). IEC comprises the *Staphylococcus* complement inhibitor protein (SCIN, encoded by the *scn* gene), chemotaxis-inhibitory protein of *Staphylococcus* (CHIPS) and staphylokinase (*sak*), which enhances the spread of the bacterium by increased proteolysis and fibrinolysis. Interestingly, an alternative version of *scn* with broad host-specificity is encoded along with the *lukPQ* gene on ϕSaeq1 (de Jong et al., 2018). The role of the human IEC is still controversial in the process of host-specific pathogenicity, since strains without the virulence gene cluster can still successfully colonize and infect the human host (Cuny et al., 2015b). It seems, however, that the emergence of IEC is rather a marker of host-adaptation of the pathogen (Matuszewska et al., 2020).

Another prophage carried virulence gene (*tarP*) was discovered recently, which encodes an alternate wall teichoic acid (WTA) glycosyltransferase, TarP. The MRSA strains expressing TarP induced remarkably less antibodies against WTA structures than those only with the housekeeping variation, TarS. Since *tarP* may also contribute to the immune evasion capacity of prominent livestock- and human associated lineages, through the modification of important cell surface antigen structures (Gerlach et al., 2018).

Tools of epidemiological investigations

Macrorestriction and pulsed-field gel electrophoresis of staphylococcal DNA

The technique is a long existing and widely used tool in epidemiological and surveillance investigations. First the bacterial genome is fragmented by a rare-cutting restriction endonuclease such as SmaI and the so obtained large DNA fragments are separated using electrophoresis. Similarity of isolates is evaluated by comparing the resultant banding patterns. Despite the many improvements and international standardization, the comparison of results among different laboratories is still difficult (Golding et al., 2015). Another problem was that some clonal lineages (e.g. clonal complex 398) had different sequence at the recognition site of SmaI and these isolates could not be typed by standard PFGE. Although the use of an alternative enzyme (Cfr9I) allowed direct comparison with SmaI–PFGE profiles, differentiation of CC398 is based nowadays mainly on *spa* and SCC_{mec} typing (Argudín et al., 2010).

Multilocus sequence typing

The population structure of the species *S. aureus* is mainly characterised by clonality, i.e., the exchange of larger genome segments between individual strains is of a negligible extent (Feil et al., 2003). Owing to this, the multilocus sequence typing (MLST), taking into consideration the

allele variants of the seven, slowly evolving household gene loci, represents a robust testing method for monitoring the descent and spread of MRSA strains (Enright et al., 2000). The strains identical in the seven alleles are assigned to the same sequence type (ST), while those identical in at least four out of the seven alleles are allocated to larger units, the so-called clonal complexes (CC). All clonal complexes have a central or 'founder' MLST variant, according to which a given isolate may differ in one, two (or more recently, three) alleles, thus such variant can be called a single locus variant (SLV) or a double locus variant (DLV), respectively (https://pubmlst.org). SLVs and DLVs mainly show genetic characteristics similar to that of the founder ST in terms of genetic plasticity, affinity for the uptake of mobile genetic elements, which are the main drivers of host specificity and adaptivity (Feil et al., 2004).

spa typing

Another widely used method is *spa* typing based on sequencing of the variable X-region of the virulence factor *Staphylococcus* protein A (*spa*) gene. The X-region is composed of variable number of *spa* repeats, which can be lost, multiply and mutate spontaneously during the bacterium's evolution (Frénay et al., 1996). In the analysis, each repeat is compared to a freely accessible, curated database, and assigned an alpha-numerical code. The *spa* type is deduced from the order of specific repeats (Harmsen et al., 2003). Despite their variability, the respective *spa* types proved to be sufficiently stable for epidemiologic typing of MRSA strains (Frénay et al., 1996).

Based on surveys simultaneously performing both MLST and *spa* typing, the determination of the *spa* type also allows classification of isolates into MLST clonal complexes (Strommenger et al., 2008). As a single-locus typing method, *spa* typing also has its limitations: in exceptional cases the same *spa* type may occur in multiple sequence types, due to large chromosomal replacement between distantly related STs, resulting in homoplasy (Price et al., 2012). Thanks to its cost-effectiveness, simple execution, and discriminatory power, by now *spa* typing has become one of the widely used typing method of *S. aureus*, in many places complementing or replacing PFGE.

Staphylococcus cassette chromosome mec (SCC$_{mec}$) typing

In addition, determining the cassette gene type of SCC$_{mec}$, carrying the methicillin-resistance gene *mec* may facilitate the differentiation of MRSA strains assigned to the same MLST or *spa* type (Kondo et al., 2007; Zhang et al., 2005).

Today the methods described above constitute the basic tools serving for the epidemiological typing of *S. aureus* and MRSA strains. The results are mostly expressed in the form of ST or CC – *spa* type – SCC$_{mec}$ cassette type, e.g. ST398–t011–SCC$_{mec}$IV.

Next generation sequencing and whole genome-based typing methods

Due to the explosive development and spread of next-generation sequencing (NGS) methods, the past two decades has brought major progress in nucleotide sequencing. With the use of these techniques, further typing methods are no longer necessary, and the data generated provide a more comprehensive and in-depth insight into the evolution and epidemiology of the different strains. A wide array of methods and bioinformatical pipelines have been developed and evaluated also for the molecular epidemiological investigation of *S. aureus*. These can be divided into two main branches, according to their approach. The gene-by-gene comparison methods apply the principle of the classical MLST on a genome-wide scale. The pipelines identify the gene loci, then compare them to a curated database and annotate the allele variants accordingly. Genetic relatedness of strains is calculated based on their shared allele variants or differing alleles, regardless of their SNP content. Results are summarized as similarity or distance matrixes, depending on the query, and similarity trees can be calculated to visualize the data. On *S. aureus* strains both whole-genome (wg) and core-genome (cg)MLST analysis can be performed: in the time of writing, there are more than 3000 described gene loci, while a subset of these, 1861 is considered and widely used as the core genome locus set, as previously defined by Leopold et al. (2014). The whole-genome based MLST approaches were tested under real epidemiological circumstances, like nosocomial outbreaks and monitoring programmes and proved to be more sufficient than any of the previously described typing techniques or their combinations (SenGupta et al., 2014).

The other branch of approaches compares the SNP content of two or more genomes and calculates the phylogenetic distance between the strains. By this methodology, more exact evolutionary relatedness can be outlined compared to the gene-by-gene techniques: the resulting trees' accuracy can be further improved by re-sampling and branching points can be supplemented with a bootstrap-value referring to their reliability. This latter type of techniques is better suited for tracing evolutionary changes, while the whole-genome based MLST methods are more appropriate for the investigation of short-term periods, like nosocomial outbreaks (Jensen et al., 2020; Price et al., 2012).

A severe hurdle of WGS-based analyses is the matter of threshold for evaluating genetic relatedness. Due to the increased resolution, every single isolate might be identified as a new type, thus the interpretation of WGS data needs more scrutiny, and a better understanding of the population structure and the evolutionary characteristics of the certain pathogen (Schürch et al., 2018).

Classification of MRSA strains

After its emergence, MRSA infection occurred primarily under hospital conditions. Although the resistance gene assortment of healthcare-associated MRSA (HA-MRSA) strains associated with nosocomial outbreaks and case number increases continuously expanded, the virulence of these strains did not change substantially: the majority of the clinical cases represented benign skin and soft tissue infections (SSTI) (Gordon et al., 2008). From the 1990s, MRSA outbreaks of a character different from the nosocomial cases were observed in many places of the world. These outbreaks shared the characteristic that the people involved had no contact with healthcare institutions and suffered from invasive infections more severe than the previously mentioned SSTIs. These novel, community-associated (CA-)MRSA strains had several properties different from those of the HA-MRSA strains. While in the case of the HA strains the SCCmec encoding methicillin resistance was of a large size (SCC_{mec} I–III), the CA strains carried smaller, novel-type cassette gene types (SCC_{mec} IV and V). Most of these strains produced a powerful virulence factor, Panton-Valentine Leukocidin (PVL), which is a dermonecrotic toxin having lytic effects on leukocytes (Gordon et al., 2008). PVL is highly responsible for invasiveness, and its presence is clearly linked to CA-MRSA strains, as it rarely occurs in HA-MRSA strains or in *S. aureus* strains sensitive to methicillin (Otto, 2013). Another characteristic of the CA-MRSA strains was that most of them remained sensitive to non-beta-lactam antibiotics (Gordon et al., 2008).

The third, epidemiologically distinct source of MRSA infections is represented by strains of animal origin (livestock-associated MRSA, LA-MRSA). In the early 1970's, Devriese (1972) described a methicillin-resistant *S. aureus* from bovine mastitis. Since then, the presence of MRSA has been documented in numerous animal species, and many domesticated species may potentially harbour not only lineages of animal origin but also those derived from humans (Cuny et al., 2015c).

Livestock-associated MRSA

Using the early phenotypic methods, strains of the *S. aureus* species were subdivided into ecovariant or ecotype groups, which were assumed to have high-level species specificity (Meyer, 2018). Similar results were obtained for MRSA strains, where the differentiation of animal and human isolates was based on early genotyping methods (e.g. PFGE) and on the antibiotic resistance profiles (Cuny et al., 2015c). However, the results of more recent comparative genetic methods have provided a much more nuanced picture (Feil et al., 2003; Turner et al., 2007). The low host specificity of certain MRSA genotypes was confirmed by the wide-ranging spread of lineages infecting numerous host species including humans, such as CC398 or CC130. The appearance of lineages originally described as human genotypes in animals, especially those kept as pets, is a source of further uncertainty (Aires-de-Sousa,

2017). For the above reasons, the justification of existence of the HA-MRSA, CA-MRSA and LA-MRSA categories is questionable (Bal et al., 2016). The human implications and potential animal host species of the most important MRSA and MSSA genetic lineages are shown in Table 1. The data presented in the table summarise only the most important results, in most cases based on cross-sectional studies; therefore, they do not allow us to draw far-reaching conclusions regarding the prolonged carriage of some less commonly isolated genotypes.

Table 1 | *Staphylococcus aureus* genetic lineages in different host species (According to Cuny, Wieler and Witte [2015] and Fitzgerald [2012]). A: sporadic occurrence; B: own observation restricted to sporadic cases in Hungary; C: occupational hazard; D: equine hospital-specific subpopulation; (genotypes marked with grey background were originally known from animal hosts); MSSA: methicillin-sensitive *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*; HA-MRSA: hospital-associated MRSA; CA-MRSA: community-associated MRSA; PVL: Panton-Valentine leukocidin

MLST clonal complex [CC], and sequence types [ST]	Human isolates	Animal hosts
CC1; ST1	MSSA, HA-MRSA, CA-MRSA, PVL+	cattle (A, B), swine, horse (A, B), chicken (MSSA)
CC5; ST5	MSSA, HA-MRSA, CA-MRSA, PVL+	horse, swine, chicken (MSSA/MRSA), turkey
CC8	MSSA, HA-MRSA, CA-MRSA, PVL+	horse, cattle, swine, cat (A)
CC9	MSSA, LA-MRSA(C)	swine (MSSA/MRSA), cattle (A), chicken
CC22	MSSA, HA-MRSA, CA-MRSA, PVL+	horse (A), cattle (B), dog, cat
CC97	MSSA(A), LA-MRSA(A)	cattle (MSSA/MRSA), swine (B)
ST121	-	rabbit
CC126	-	cattle
CC130	MRSA (A, B)	cattle, sheep, horse (A, B), dog, cat
CC133	-	sheep (MSSA), goat (MSSA), cattle (MSSA), wild boar (MSSA)
CC705	-	cattle
CC385	-	chicken, wild birds
CC398, ST398 (human-adapted subpopulation)	MSSA, MRSA, PVL+	-
CC398, ST398 (animal-adapted subpopulation)	MSSA, LA-MRSA (C)	swine (MSSA/MRSA), cattle (B), small ruminants, chicken, turkey, rabbit, horse (D), dog, cat
ST425	LA-MRSA (A)	cattle, wild boar (MSSA) (A)
ST1464	-	sheep

Emergence of MRSA in swine and cattle herds

The emergence of LA-MRSA CC398 in pigs

The documented history of pig-related MRSA begins with the first reports of pig to human transmission of strains in the early 2000s. In 2005, after investigating the source of some previous human clinical cases, a more than 760-times greater prevalence rate of MRSA

carriage was found among Dutch pig farmers, compared to patients admitted to local hospitals. (Voss et al., 2005) In that times, The Netherlands was known as a country with <1% MRSA prevalence rates, both regarding clinical isolates and patients admitted to hospitals, one of the lowest in Europe, which even more emphasized the severity of the problem. The isolated MRSA strains were not typeable (NT) by PFGE analysis, and showed a that time rare sequence type, ST398.

Prevalence and population structure

After this lineage was found to be widespread in the Dutch swine sector, some other European countries became concerned and by investigating their own situation and reported the presence of the pathogen both in livestock and humans (Lewis et al., 2008; Witte et al., 2007). Finally, these findings motivated the European Food Safety Authority to propose and co-ordinate a multinational European baseline survey in 2008, with the participation of more than 5000 holdings (breeding and production holdings) from 26 countries, to investigate MRSA in pigs. Farms in 17 countries were tested MRSA-positive, and the average prevalence of positive breeding and production holdings was 14.0%, and 26.9%, respectively. However, some countries were apparently free of pig-related MRSA, a few countries highly exceeded the average prevalence rates, like Spain (46.0% and 50.2%), Germany (43.5%, and 37.4%) and Italy (14.0%, and 33.9%) regarding breeding and production holdings, respectively. The majority of isolates belonged to three *spa* types t011, t034 and t108, all related to CC398. Other variants were identified with lower frequencies.

In the following years, a few countries conducted further screening programmes to monitor the changes of MRSA prevalence in their swine sector, with varying results. In most countries, the prevalence rates generally increased, along with the genetic variability of the isolates (Mroczkowska et al., 2017; Peeters et al., 2015). Denmark, however, beside a dramatic prevalence boom, reported a more homogenous clonal population of MRSA CC398 strains in 2014 compared to 2008 (Sieber et al., 2018). The whole genome-based phylogenetic analysis of more than 200 isolates from these studies later showed, that the increasing prevalence was caused by clonal expansion of three dominant lineages (termed L1, L2 and L3), including most t034 and t011 strains. As a driver mechanism, animal movements have suggested to play a critical role in the dissemination of the pathogen. The positive correlation between the number of animal movements, MRSA-status and genetic relatedness of isolates among trading farms reinforced this assumption. It is worth noting, that the Danish swine sector shows a very pyramidal structure, in which a few nucleus and multiplier holdings supplies the numerous other production and weaner farms with breeding animals, since the distribution and isolation frequency of some lineages has a rather country-specific explanation as well (Sieber et al., 2018). In the last decade, a plethora of studies aimed to investigate the epidemiology of MRSA

in livestock, especially in the swine sector, to better understand the risk factors and dynamics of transmission and find better preventive and control measures.

Risk factors, introduction, and transmission of MRSA within swine herds

According to the EFSA baseline survey, main risk factors for introduction and persistence of MRSA in swine herds are herd sizes and breeding animal replacement practices (2010). Larger herds seem more likely to be MRSA-positive, since larger farms, compared to smaller ones, may have a higher risk introducing MRSA-positive animals trough trading and have a larger population of susceptible animals, which means a higher probability of persistence over longer periods (Broens et al., 2011). The weak, but significant correlation between the probability of MRSA-positivity and the replacement policy of breeding animals (gilts mainly own bred or purchased), was also due to the effect of purchasing animals from positive herds, as later results suggested (Espinosa-Gongora et al., 2012). Similarly, herd size and herd type appeared to be a crucial risk factor of spreading MRSA among herds in the case of fattening holdings (Alt et al., 2011; Fromm et al., 2014). Of note, additional factors should also contribute to the spread of MRSA in the case of closed herds with high hygiene status (Alt et al., 2011; Grøntvedt et al., 2016). Extreme carriage rates among pig farmers and swine veterinarians is a well-known phenomenon (Voss et al., 2005), and these professionals can play a role in introducing the pathogen into negative herds (Grøntvedt et al., 2016). Airborne contamination is another possibility in regions with high swine farm density, since viable MRSA could be detected in the downwind of stalls, 150-200 meters from the buildings (Schulz et al., 2012; Ferguson et al., 2016).

Within a herd, MRSA is spread predominantly by direct contact between animals (Broens et al. 2012; Bangerter et al. 2016). However, most pigs change MRSA status several times during their lifetime, which implies that carriers are being transiently rather than permanently colonised (Bangerter et al., 2016). In contrast, once a herd becomes MRSA-positive, the status will hardly change (Peeters et al., 2015), since dust, contaminated surfaces, feed (Ferguson et al., 2016) and even already colonised farmers and veterinarians may serve as reservoirs for MRSA (Köck et al., 2012).

Given the multidrug-resistant phenotype of most isolates, the use of antibiotics is an evident factor to be associated with the emergence and transmission of MRSA in swine. Especially group treatments with antimicrobial drugs and heavy metals provides a strong selective advantage for MRSA to persist and spread (van Duijkeren et al., 2008). The matter deserves a special attention on a broader perspective for more reasons. First, most resistance genes in staphylococci are encoded on mobile genetic elements (such as plasmids, transposons,

cassette chromosomes), of which some can transfer resistance not only within the genera but among other Gram-positive pathogens of animals and humans as well (Schwarz et al., 2014; 2018). Second, various mobile genetic elements often carry more resistance genes, thus using one compound unwillingly co-selects resistance towards other antimicrobials too (Schwarz et al., 2014). At last, the matter is further complicated with the persistence of multi-resistance genes conferring resistance towards more compounds from diverse antibiotic classes. One prominent example of these is the plasmid-borne *cfr* gene, which simultaneously codes resistance for phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics (Long et al., 2006). In this work, the matter of resistance is further discussed from microevolutionary and not from clinical aspects.

Clinical relevance of MRSA in swine

Despite the high MRSA carriage rates, the role of MRSA in clinical infections is, at least, argued. Sporadic reports of infections of the skin, the urogenital tract and mammary gland, as well as from joints and septicaemia have been described (Schwarz et al., 2008; Kadlec et al., 2009; Meemken et al., 2010). Van Duijkeren et al. (2007) described cases of exudative epidermitis in 2006, where MRSA CC398 could be isolated in pure cultures, and *Staphylococcus hyicus* was absent. A similar outbreak of exudative epidermitis was reported by other from three pig farms in Portugal (Pomba et al., 2010). The infections involved around 15% of the weaned piglets and resulted in an additional 20% mortality rate. Besides skin infections, MRSA can cause a variety of lesions. In a collection of *S. aureus* strains collected over a 4-years period and originating from pathological lesions in pigs Meemken et al. (2010) found, that more than 40% of the *S. aureus* isolates were MRSA. The vast majority of these (95%) were MRSA CC398; and in 35% of the cases, MRSA was the only pathogen that could be cultured from the lesion, and thus considered as the primary causative agent. The incidence of MRSA-positive lesions did not change over the oncoming four years, contradicting the previously expected emergence of CC398 cases (Voss et al., 2005; van Loo et al., 2007).

A similar tendency was observed in another study, where *S. aureus* (both MSSA and MRSA) was isolated with equally low proportions per year over a 6-years period (van der Wolf et al., 2012). From 22,608 swine samples submitted for post-mortem examination, only 144 (0.6%) *S. aureus*-positive cases were reported. Of these, 116 isolates were included in the study for further investigation. From this 116 *S. aureus* isolates only 16.3% were MRSA and 9,5% were reported as the primary cause of infection, however, except one ST1–t127 isolate, 18 strains belonged to the CC398 lineage. According to these results, the clinical relevance of MRSA in swine health seems to be relatively subtle.

LA-MRSA in dairy cattle

In dairy cattle, *S. aureus* is considered as one of the most important bacteria causing contagious clinical or subclinical intramammary infection (IMI), which contribute to reduced milk quality, significant milk loss, and may result in chronic infection. According to recent studies, the genetic characteristics of *S. aureus* influence both severity and prevalence of the infection within dairy herds. (Cremonesi et al., 2015; Haveri et al., 2007; Lundberg et al., 2014) A small number of clonal types, like the sequence types of CC97 and CC151, are associated with majority cases of bovine mastitis worldwide. Most of these strains also seem to be more virulent than others, as often they harbour additional virulence factors: exfoliative toxins, enterotoxins, and the bovine specific bi-component leukocidin, lukMF'. (Thomas et al., 2021) Accordingly, these strains are frequently isolated from udders affected with IMI, while other lineages may co-exist with the major variants within a herd, but with far less clinical relevance. (Smith et al., 2005; Thomas et al., 2021) Methicillin-resistant *S. aureus* was first described in 1972 from a bovine mastitis case (Devriese, 1972). Nevertheless, mastitis of MRSA origin is an occasional observation and rarely causes a stock-level problem (Aires-de-Sousa 2017).

In Hungary, between 2001 and 2002, Kaszanyitzky and colleagues tested nearly 2,000 *S. aureus* strains isolated from various animal species and foods, and nearly half of the samples examined were of udder or cow's milk origin. However, only 5 of the strains were found to be MRSA. The 5 MRSA strains were derived from two dairy herds. (Kaszanyitzky et al. 2004). In 2006, Peles et al. (2007) could not isolate any MRSA from bulk tank milk samples of 20 dairy farms. A third Hungarian publication from 2007 reports MRSA strains isolated from subclinical mastitis in a dairy farm. The ST1–t127 genotype was carried by all affected animals and one worker (Juhász-Kaszanyitzky et al. 2007). Cases similar to the domestic observation have become common in Italy (Alba et al. 2015), and further data support the low species specificity of the genotype (Cuny, & Witte 2015). Other common genotypes in Europe are CC8, CC97, CC130, and CC398 (Cuny et al. 2010; Fitzgerald 2012).

Livestock associated MRSA in humans

The mainly swine-associated MRSA CC398 is concerned as a potential human pathogen, as first reports of human clinical cases were reported from Western-Europe (Lewis et al., 2008; Declercq et al., 2008). To date it seems, that infections remained sporadic, and although the pathogen can result in severe clinical outcome, most cases remained benign with less complications compared to other lineages (Becker et al., 2017).

Unlike clinically apparent infection, carriage of MRSA CC398 may be common, and it is primarily an occupational hazard in people working in animal husbandry and especially in the pig industry (Cuny et al., 2015c; Walter et al., 2016). In some cases, the carriage rate of MRSA CC398 may reach 60–80%, both among pig farm workers and the attending veterinarians of the farms (Reynaga et al., 2016; Van den Broek et al., 2009). In most cases, carriage is temporary, and in

people not exposed to the contaminated area or animals (e.g., during vacation) spontaneous decolonisation takes place within a short time (Walter et al., 2017).

Equine specific MRSA strains in horses and humans

MRSA genotypes in equine hospitals and related horse population

Most MRSA infections in horses can be linked to cases occurring in equine hospitals. MRSA infection in horses was first described in the United States in 1997, and then in the very same year in Japan (Hartmann et al., 1997; Shimizu et al., 1997). The report on the first MRSA outbreak in an equine hospital was published two years later (Seguin et al., 1999). While in Canada and the United States sequence types corresponding to those of the human hospital strains (ST8) were identified (Weese et al., 2006), in Europe the sequence type ST389, previously associated with pigs, became dominant (Cuny et al., 2017). This latter sequence type was first detected at the Equine Clinic of the University of Veterinary Medicine in Vienna (Cuny et al., 2008), and subsequently it was isolated at equine clinics in the Netherlands (van Duijkeren et al., 2008), Switzerland (Panchaud et al., 2010; Sieber et al., 2011), Germany (Vincze et al., 2014), Belgium (Van den Eede et al., 2009), and Spain (Gómez-Sanz et al., 2014). The sequence type proved to be extremely successful: within less than a decade starting from the early 2000s, it has replaced other, previously important MRSA genotypes (Cuny et al., 2008; Loncaric et al., 2014) or appeared as a new pathogen at equine clinics of the continent, acquiring a permanent presence at the affected places.

The ST398 MRSA strains isolated in equine hospitals share numerous traits, which is indicative of their common origin: the majority belong to *spa* type t011, they have SCC_{mec} type IV cassette chromosome, and their broad-spectrum phenotypic aminoglycoside (kanamycin, gentamicin, tobramicin) resistance is encoded by the *aac(6')-Ie-aph(2")-Ia* bifunctional resistance gene. In addition to the aforementioned genes, horse-specific versions of several staphylococcal virulence genes are also known, such as the von Willebrand factor-binding protein (vWbp) playing a role in blood coagulation, the staphylococcal complement inhibitor protein (SCIN) and LukPQ, a toxin similar to Panton-Valentine leukocidin (Koop et al., 2017; Viana et al., 2010). The fact that their appearance is restricted to strains of equine origin proves a higher degree of adaptation to the host species within the ST398 genotype (Walther et al., 2018). By in-depth analysis of the genome of MRSA CC398 strains of equine hospital, pig and human origin, Abdelbary et al. (2014) have identified some point mutations typical of only the strains isolated from equine hospital cases, on the basis of which the latter can be distinguished from other members of the ST398 sequence type. One of them, named canSNP1748T was investigated on a large collection of strains by a specific PCR test and reinforced the specificity of the SNP (Cuny et al., 2015b). Other investigations also suggest that this narrow group of

ST398 is responsible for the majority of case number increases and outbreaks in equine hospitals across the European continent (Cuny et al., 2017).
From the epidemiological point of view, the disease outbreaks occurring in equine hospitals resemble the human hospital cases: the strains persist in the equine hospital environment for several years, and their spread is mainly facilitated by fomites and the personnel (van Balen et al., 2014; Hoet et al., 2011). At the same time, outside the hospital environment the strains occurring in equine clinics can only sporadically be found in the horse population as well as in the human population in contact with horses (Van den Eede et al., 2013; Van den Eede et al., 2012; Guérin et al., 2017). The hospital character of infections caused by MRSA CC398 of equine origin is also supported by the extensive antibiotic resistance profile: like the HA-MRSA isolates, the strains occurring in equine hospitals are often resistant to all the antibiotics that can be used in equine medicine.

In addition to the equine hospital subpopulation of MRSA ST398, rarely the representatives of other genotypes may also cause hospital outbreaks or isolated not associated with hospitals. In Europe, the strains belonging to the previously mentioned ST8 sequence type caused hospital outbreaks first in Ireland (O'Mahony et al., 2005) and subsequently in the Netherlands and Germany. In the latter two cases, however, they were gradually replaced by CC398 strains, and thus their isolation rate decreased to 1–2% (Cuny et al., 2017; van Duijkeren et al., 2010). Strains of genotype ST254, detected in several equine hospitals of Europe at the beginning of the 2000s and closely related to the ST8 sequence type, may also occur occasionally (Cuny et al., 2008; Walther et al., 2009), but their significance is negligible (Vincze et al., 2014). Strains of sequence type ST1–t127, which can be isolated from numerous animal species and, also from humans, constitute a special group of the cases associated with horses. Because of its broad host range, the presence of this genotype in horses also needs careful attention. Within Europe, it is common primarily in livestock in Italy (Alba et al., 2015; EFSA, 2009), while the number of cases caused by it in equine hospitals is insignificant (Cuny et al., 2008; Loncaric et al., 2014; Sieber et al., 2011). In Hungary, strains of ST1–t127 genotype have so far been detected only in cattle and in farm workers dealing with them (Juhász-Kaszanyitzky et al., 2007), as previously mentioned.

The clinical significance of MRSA in horses

In horses, the outcome of contamination with MRSA may vary from the asymptomatic carriage to fatal invasive infection. The commonly occurring syndromes include skin and soft tissue infections, osteomyelitis, implant-associated infections, metritis, omphalitis, thrombophlebitis and pneumonia (Anderson et al., 2009). Experience shows that horses passing through the infection may become persistent carriers, although so far there has been only a single

publication to support this. In a follow-up study conducted in Sweden on 15 horses, the duration of post-infection carriage varied between 55 and 711 days, the median value being 143 days, which is in good agreement with the 6-month period observed in studies on humans (Larsson et al., 2011). To date, no data are available about the proportion of potentially contaminated animals released from equine hospitals affected by MRSA or about the time needed for their natural decontamination or decolonisation. Thus, we do not know the extent of the risk that the re-entry of horses released from equine hospitals into studs and liveries without a quarantine period may pose.

If MRSA becomes endemic in a horse stud, the issue of eradication may also arise. Based on the single publication available on the subject, decolonisation of asymptomatic horses could be achieved by the introduction of hygienic measures alone. The time needed for achieving MRSA-negative status for individual horses varied between 2 and 4 months (Weese et al., 2005). In human medicine, the decolonisation of carriers by medicines is a well-established practice, and thus studies on the natural elimination of the pathogen are not feasible. At the same time, the success rate of decolonisation therapy is only around 40% according to the results of the relevant studies (Harbarth et al., 1999; Sai et al., 2015). In equine medicine, little is known about the efficacy of a potential decolonisation therapy. In the above-cited Canadian study, prolonged oral chloramphenicol therapy of a horse was attempted twice without success, despite the in vitro sensitivity of the involved MRSA strain to that antimicrobial agent. Eventually, 30 days after the last treatment, the horse became decolonised spontaneously in the sixth month of the study (Weese et al., 2005). Based on the above findings, the use of antibiotics for decolonisation purposes should be a ‘last resort’ option because of its doubtful outcome, the rapid acquisition of antimicrobial resistance by the strains and the possible complications of prolonged antibiotic therapy.

Occurrence and significance of MRSA strains of equine origin in humans

Whether or not an MRSA strain can colonise humans depends on the host specificity of the given genetic lineage, while its pathogenicity is determined by its virulence genes mainly encoded by mobile genetic elements (Turner et al., 2007). The majority of genotypes hitherto isolated from horses belong to genetic lineages having a broad host range, with the group of strains belonging to clonal complex 398 being the most outstanding. The genotype group was a methicillin-sensitive pathogen of human origin. Its colonisation of domestic animals was accompanied by the loss of a substantial proportion of virulence genes of human origin and the acquisition of the gene segment coding for methicillin resistance (Price et al., 2012). For further details, please see later in this section. It can be explained by the former that the bacteria belonging to this genotype are still capable of permanently colonising humans, while human-to-human cross-contamination has been extremely rare so far (Würtz et al., 2017), and

these bacteria rarely cause human infection. Out of the 13,756 MRSA strains analysed in a survey conducted by the European Centre for Disease Prevention and Control in 2013, encompassing 27 European countries, 3.9% belonged to the CC398 genetic lineage (Kinross et al., 2017), while in a similar study performed in Germany 6.1% of the 5,500 MRSA isolates were found to be members of that lineage. In the latter case, the proportion of the equine-specific CC398 subpopulation was only 0.1% (6 isolates), while two strains belonging to other horse-associated genotypes were isolated. A similar result was obtained by the analysis of 10,864 MRSA isolates originating from human clinical samples, among which the proportion of strains belonging to a genotype associated with horses was found to be 0.14% (Cuny et al., 2015b).

Prevalence of carriage among humans working as equine professionals

Professionals working with horses have a lower MRSA carriage rate, as compared to pig farm workers (60-80%). Based on the results of several studies, the MRSA carriage rate of people working in an equine hospital setting varies between 9.4 and 27.8%, and from their samples almost exclusively the genotypes present in the equine clinic can be identified (van Duijkeren et al., 2010; Sieber et al., 2011). Like in the pig industry, the proportion of colonised people is considerably higher among those that are regularly in contact with horses and their direct environment (veterinary internists, assistants) (van Duijkeren et al., 2010).

Veterinarians, outside the equine hospital setting, also show similar prevalence values. According to a paper presented at an equine medicine conference held in Canada in 2008, 10.1% of 257 veterinarians sampled (26 persons) were found to be MRSA positive, the majority of them carrying a genotype of animal origin (Anderson et al., 2008). As stated previously, in the horse populations outside equine clinics low prevalence values can be measured, which explains the low rate of MRSA burden found in people pursuing equestrian sports. In a survey simultaneously examining horses and their riders, out of 166 couples only 4 persons and two horses belonging to them proved to be MRSA positive. In both cases, the horses and their riders carried the same CC398 genotype strain. It is important to mention that two out of the four persons worked in a highly busy equine hospital and one of them as a dog breeder at the time of the study (Van den Eede et al., 2013). Because of this fact, and due to the possibility of human-to-animal cross contamination, biosecurity should be a priority also in the equine practices, with special attention to personal hygiene when treating patients at risk of MRSA infection (Koop, 2016).

Origin and evolution of CC398 LA-MRSA in Europe

Little is known about the prevalence and evolution of MRSA CC398 before the early 2000s, when it was first recorded and started spreading rapidly among pigs in Europe. The retrospective investigation performed by Espinosa-Gongora et al. (2014) revealed an apparent lack of the clonal lineage among swine *S. aureus* isolates in the timeframe of 1973-2003, suggesting a young history of MRSA CC398 in this species. Other results provided a plausible explanation for this finding and indicated a recent host jump of the CC398 strains from human to animals. By the microevolutionary investigation of 89 *S. aureus* CC398 whole genomes, both of human and animal origin, Price et al. (2012) could identify the human MSSA CC398 strains grouped into the most ancestral clade. The authors proposed, that the originally methicillin sensitive strains of the clonal lineage jumped and adapted to the animal hosts. The process was resulted in the loss of some human-associated genetic traits, like the immune-evasion cluster genes and gain of the methicillin, tetracycline, and zinc resistance in return. Hence the extensive use of tetracyclines and zinc in the swine industry could highly contribute to the selection and drastic spread of the pathogen mainly in pigs but in other livestock species too, as it was reinforced by the results of others (Cavaco et al., 2011; Slifierz et al., 2015).

As it was previously outlined, the first equine-related MRSA CC398 infections and nosocomial endemics were also reported in the 2000's, in parallel with the increased spread of other CC398 lineages (Cuny et al. 2006). However, the equine isolates showed more consistent characteristics, as they were and still are almost exclusively *spa* type t011, SCC_{mec}IV and harbour the bicomponent aminoglycoside resistance gene ***aac(6')Ie-aph(2")Ia*** and some lineage-specific SNPs (Cuny et al., 2017). A horse-specific leukocidin gene *lukPQ* was also described in other, horse-specific *S. aureus* clonal lineages and subsequently was reported in some horse-related CC398 isolates (Walther et al., 2018), suggesting a further adaptation step of this sub-clone to the horse population.

Considering their evolutionary origin, the emergence and circulation of LA-MRSA CC398 strains in humans without livestock contact is only part of a re-adaptation process. The genomic investigation of a large set of LA-MRSA CC398 isolates in Denmark revealed mostly recent transmission of strains from livestock to humans, though with much lower frequencies, livestock-independent transmission pathways also were identified (Sieber et al., 2019). These strains lost some resistance determinants and re-gained human-associated virulence genes, probably to better fit the changed selective pressure meant by the new host environment. Regardless of whether the clonal lineage might re-gain a wider access to the human host or healthcare-associated cases remain sporadic and strictly associated with livestock environment, the pathogen shows a marked genomic plasticity and adaptivity. Hence, the importance and risk of LA-MRSA CC398 should be monitored and re-evaluated on a regular basis.

3. Aims of the study

Since its first discovery in the early 2000s, studies assessing the prevalence and zoonotic potential of the LA-MRSA CC398 lineages were widely published from most part of Europe. While in Hungary, recent data on the occurrence and prevalence rates of LA-MRSA in different large animal species are still scarce. Hence, our **first goal was to investigate the occurrence and population genetics of MRSA in swine which is an important reservoir of LA-MRSA CC398** strains, and in other selected species in which staphylococci are considered as major pathogens (dairy cattle and horses).

Besides their veterinary importance, these strains pose potential human medical risk in terms of their capacity to colonise and, in some instances, cause life-threating infections in humans. The re-adaptation of this lineage to the human host has already been suggested, as some human originated LA-MRSA isolates harbour human-associated genetic traits. Our **second aim was to assess the zoonotic potential of the recovered LA-MRSA CC398 isolates** by comparing animal and human originated strains, using an array of molecular epidemiologic tools.

Though LA-MRSA in the human population seems to be not significant yet, the emergence of new resistance mechanisms poses a further risk in the case of pathogens which can colonise several host species, like *S. aureus*. The overlap between the antimicrobial usage of human and veterinary medicine may give rise to the emergence of antimicrobial resistance genes between the different host species, through the interspecies colonisation with the pathogen, and horizontal gene transfer within the microbiota. Thus, **the third aim was to investigate the antimicrobial resistance profiles** of the isolates, focusing on resistances which are encoded on mobile genetic elements.

A special attention was dedicated to horses, in which species LA-MRSA CC398 emerged mainly as a nosocomial pathogen in Europe and means a severe challenge to veterinary medicine. Despite the relative long persistence of certain lineages in the European equine clinics, the occurrence and genetic relatedness of the Hungarian clinical and sub-clinically carried strains are yet not discovered. Our **fourth goal was to investigate the equine-related CC398 strains isolated in a 10-years period** in and outside the Department and Clinic of Equine Medicine, to have a better understanding of the clinic's role in the Hungarian equine-related MRSA epidemiology.

4. Materials and methods

4.1 Swine industry-related MRSA isolates

4.1.1 Origin of swine industry-related samples

One hundred of the total 292 large-scale breeding and production farms with more than 100 sows were randomly selected across the country and invited to participate in the survey. This sample size was determined to be able to estimate the proportion of MRSA-infected farms. The Epitools package (available at https://epitools.ausvet.com.au/oneproportion) was used for this calculation, with an estimated proportion (target prevalence) of 0.7 (70%), a desired precision of the estimate of 0.1 (10%), and a 95% confidence interval of the estimate. The calculation suggested the random selection of 64 farms to reach the goals of the sampling. The 100 figure was used instead to allow for loss of farms due to refusal to participate (meaning a 64% response rate). Finally, two randomly chosen fattening units were also invited to complete the survey. Hence, the number of investigated farms was 40.

Environmental dust samples were collected between May 2019 and December 2019 from five different production units on each farm, whenever possible, representing more age or production groups. Samplers were asked to rub a minimum 0.5 m^2 area per unit with a 5 × 5 cm sterile dry cotton swab. Swabs were then pooled in a sterile plastic bag and delivered to the laboratory at ambient temperature (2–25 °C) within 10 days. A questionnaire was supplied to inquire about the basic farm-related data, the primary source of the breeding population, regular animal movements between farms, and information on previous occurrences of MRSA. The completed questionnaire and a signed consent form were mandatory for further processing of the samples. Veterinarians and farm managers (henceforth: swine professionals) of the respective farms could also provide a sample voluntarily. Human sampling was performed by wiping both nares with a cotton tipped swab, which was then placed into Amies transport medium containing charcoal. Human swabs were shipped and processed along with the environmental samples of the respective farm.

In accordance with the Act XXVIII of 1998 and Government Decree 40/2013 (II. 14) the environmental sampling of swine holdings is not defined as animal study and thus it does not require further legal or ethical permission. All sampled swine professional has participated voluntarily in the survey and provided an informed written consent. The human sampling protocol of swine professionals was supervised and approved by the Committee of Science and Research Ethics, Medical Research Council, Ministry of Human Capacities, Hungary, as a protocol that does not require medical intervention (No. 42323-2/2019/EKU).

4.1.2 Culturing the bacteria

Upon their arrival, environmental swabs were incubated overnight (16–20 h) at 37 °C in 100 ml Mueller-Hinton broth supplemented with 6.5% (w/v) NaCl. Subsequently, about 10 µl of the

enrichment suspension was spread simultaneously onto chromogenic agar plates for the selection of *S. aureus* (BD BBL CHROMagar Staph aureus) and MRSA (BD BBL CHROMagar MRSA II, Diagon Ltd, Hungary) and incubated at 35 ± 1 °C for 24–48 h. One colony per sample, showing the characteristics described by the manufacturer, was chosen from the MRSA selective medium and sub-cultured on Columbia sheep blood agar plates under the same conditions. If more than one phenotypic variant of presumptive MRSA was present on the chromogenic agar plate, then one colony of each variant was chosen. Human nasal swabs were processed the same way. Colonies from the *S. aureus*–selective plates were treated as described above, only if the MRSA medium yielded no isolates. Pure cultures of the bacteria were stored at -80 °C until further investigation.

4.1.3 Molecular investigations and antibiotic susceptibility testing

The initial identification of presumptive *S. aureus* and MRSA isolates included a multiplex polymerase chain reaction (PCR) targeting the *spa* gene, a species-specific marker of *S. aureus*; the *mecA* and *mecC* genes, confirming methicillin resistance; and the *lukS-PV/lukF-PV* gene, the marker of the human-related Panton-Valentine leukocidin virulence factor (Stegger et al., 2012). Only MRSA strains were characterised further.

The minimal inhibitory concentrations (MIC) of 19 selected antibiotics were determined by microdilution in Mueller-Hinton broth using the Sensititre EUST plates (Thermo Fischer Scientific). When applicable, MIC values were interpretated using the European Committee on Antibiotic Susceptibility Testing (EUCAST, 2021) criteria. In the case of sulfamethoxazole, the Clinical and Laboratory Standards Institute (CLSI, 2021) criteria were applied. Epidemiological cut-off values were used to determine the wild-type susceptibility to kanamycin, mupirocin, streptomycin, and tiamulin according to the methodology recommended by the European Union Reference Laboratory for Antimicrobial Resistance (EFSA & European Centre for Disease Prevention and Control [ECDC], 2018).

4.1.4 Whole genome sequencing and genomic analysis of strains

Fifty-seven isolates were selected for whole-genome sequencing (WGS). Whole-genome sequences were obtained from a NEBNext Ultra II directional DNA library with TruSeq adapters on an Illumina NovaSeq 6000 sequencing system (2 × 150-bp paired-end reads) at the NGS Platform, University of Bern, Switzerland. The resulting Illumina reads were transferred to BIOMI Kft., Gödöllő, Hungary, for further bioinformatic investigation. Whole-genome multilocus sequence typing (MLST) analysis was performed on assembly free (AF) datasets using the BioNumerics software package version 8.0 (Applied Maths NV, Belgium), as described previously (Német et al., 2020). Then distance matrices were generated by selecting the subset of 1861 core genome (cg) loci of each isolate. To visualise genetic relatedness, unweighted pair group method with arithmetic mean (UPGMA) trees were constructed based on the cgMLST allele matrices. No resampling was performed during the tree construction, and branch lengths

were calculated according to the average allelic differences of the isolates. The trees were annotated based on the iTOL 6.3 online platform (Letunic et al., 2021). Clusters of closely related isolates were defined according to previous recommendations, using a 24 allelic difference as a cut-off value (Schürch et al., 2018). Recent transmission events were considered if the pairwise allelic difference was lower between the two strains than the estimated median annual variability within a *S. aureus* population (≤ 5) (Lagos et al., 2022). *spa* typing and classical MLST of the seven housekeeping gene loci was performed within BioNumerics. De novo draft genome contigs were assembled using SPAdes in BioNumerics (Prjibelski et al., 2020), and RASTtk was used for the annotation of selected genomes (Brettin et al., 2015).

Resistance genes and mutations generating antibiotic resistance and the mec-carrying cassette chromosome (SCCmec) type were investigated by using the online ResFinder 4.1 (Joensen et al., 2014) and SCCmecFinder 1.2 (Kaya et al., 2018) tools with default settings (threshold of coverage: 60%, threshold of identity: 90%). The presence of virulence genes was assessed by using the sequence extraction tool in BioNumerics and confirmed by mapping the assembled genomes against the given virulence gene as reference in Geneious Prime 2022.1.1 (Biomatters Ltd., New Zealand) with default settings. Hits of 100% coverage and > 98% nucleotide identity were considered valid results. The homology of the annotated draft genomes to plasmids known to carry the *cfr* resistance gene, pSCFS3 (AJ879565.1) and pSA737 (KC206006.1), was investigated in the same way. The genetic vicinity of the identified cfr gene was also investigated by using Geneious Prime.

Further 14 human-derived MRSA CC398 isolates, from 2019, were involved in the study. The MRSA isolates obtained from human clinical samples were routinely submitted to the AMR NRL of the National Public Health Centre for molecular typing between January and December 2019. Strains were selected based on their non-typeability (NT) by SmaI-pulsed-field gel electrophoresis (PFGE) and *spa* type related to CC398. WGS was performed in the sequencing facility of the National Public Health Centre on the MiSeq platform (Illumina) using 150-bp paired-end chemistry. Raw reads were then analysed at BIOMI Kft. as described previously. WGS data from this study are available in the Sequence Read Archive under the project numbers PRJNA901421 and PRJNA893357.

Whole-genome data of nine LA-MRSA strains from Denmark were included in the cgMLST analysis for comparative purposes. The strains represent the three dominant CC398 lineages – L1, L2, and L3 – as identified by Sieber et al. (2018). To reinforce the cgMLST results in the case of closely related isolates, single nucleotide polymorphism (SNP) analysis was performed by using an in-house pipeline on all 79 strains. The pipeline is detailed in Supporting Information S1. A maximum likelihood tree was generated and then visualised and annotated with iTOL.

4.2 Occurrence and characterisation of methicillin-resistant *Staphylococcus aureus* isolated from bovine milk

4.2.1 Origin of isolates

To investigate the genetic variability of MRSA isolates from milk of a 15-year timeframe, conserved MRSA strains of the Veterinary Diagnostic Directorate (VDD), National Food Chain Safety Office, Budapest were characterised further. Presumptive *S. aureus* isolates from two large-scale milk hygiene laboratories [University of Veterinary Medicine Budapest (UVMB), Hungary and Livestock Performance Testing Ltd., Gödöllő, Hungary (LPT Ltd.)] were collected prospectively between July 2017 and December 2018.

4.2.2 Molecular identification and genotyping of isolates

All prospectively collected, presumptive *S. aureus* strains were tested by mPCR-1 for *spa*, *mecA*, *mecC* and *lukS-PV/lukF-PV*, as described in Section 4.1.3. *spa* typing was performed by sequencing the resultant *spa* gene amplicons with the same primers used in the multiplex PCR (spa-1113f and spa-1514r) in the sequencing facility of the BIOMI Kft. (Gödöllő, Hungary) as described by Stegger et al. (2012). Conserved MRSA strains from the VDD were tested in the same way. Multilocus sequence typing (MLST) was carried out on selected representative strains of each *spa*-type (Enright et al., 2000) also at the BIOMI Kft. MLST sequence types and *spa*-types were analysed using the corresponding plugin of the BioNumerics v7.6 software (Applied Maths NV, Belgium).

4.2.3 Antimicrobial susceptibility testing and resistance genes

Antimicrobial susceptibility testing (AST) was performed on representative MRSA *spa*-types from each farm (n = 14). Susceptibility was determined by using the agar disc diffusion method and evaluated according to the standards of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for *S. aureus* (EUCAST, 2019). In the case of streptomycin and tiamulin, there were no zone diameters available that were approved by EUCAST or Clinical Laboratory Standard Institute (CLSI) standards. Thus, besides phenotypic testing, streptomycin resistance was further evaluated for the presence of a resistance mechanism by investigating *aadE* and *str* genes in all phenotypically tested isolates (Klare et al., 2007; Schijffelen et al., 2010). Both genes are known to confer resistance towards streptomycin in staphylococci (Wendlandt et al., 2013). In the case of tiamulin, the previously proposed and published zone diameters were used (Jones et al., 2002). The 17 antimicrobials were as follows: chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), clindamycin (DA, 2 µg), erythromycin (E, 15 µg), fusidic acid (FA, 10 µg), gentamicin (CN, 10 µg), kanamycin (K, 30 µg), linezolid (LNZ, 10 µg), penicillin (P, 10 µg), quinupristin-dalfopristin (SYN, 15 µg), rifampin (RA, 5 µg), streptomycin (S, 10 µg), sulphamethoxazole-trimethoprim (STX, 25 µg), tetracycline (TE, 30 µg), tiamulin (TIA, 30 µg), tigecycline (TGC, 15 µg) and tobramycin (TOB,

10 µg). Additionally, the genetic determinants of tetracycline resistance *tet*(K), *tet*(M) were amplified with primers tetK-1/tetK-2, and tetM-1/tetM-2, respectively (Birgit Strommenger et al., 2003), while *tet*(L) was detected using primers tet(L)-2-1 and tet(L)-2-2, described by Aarestrup et al. (2000). Aminoglycoside-modifying enzyme genes *aacA-aphD*, *aphA3* and *aadD* were detected by a multiplex PCR (Schmitz et al., 1998).

4.3 Investigating MRSA isolated from horses and related humans in Hungary

4.3.1 Characterisation of MRSA strains isolated in the Department and Clinic of Equine Medicine from two outbreaks between 2011 and 2016

4.3.1.1 Origin of isolates

Samples were continuously taken from horses hospitalized in the DCEM for routine bacteriological culture between July 2011 and May 2016 and isolates which represented cefoxitin-resistant, haemolytic *Staphylococcus* sp. were considered into this study (2015). Sampling sites where cefoxitin-resistant, haemolytic *Staphylococcus* spp. were found included postoperative wound infections (n=22), abscesses (n=3), thrombophlebitis (n=3), postoperative intraabdominal infections (n=2), conjunctival infections (n=2), pleuritis and pneumonia (n=2) and routine nasal swabs or tracheal wash samples (n=9). In October 2017, a voluntary screening was performed on the clinic personnel to investigate the potential occupational risk groups of nasal carriage. Samples were collected from 36 out of 39 people, including veterinary internists (n=6), surgeons (n=6), technicians (n=15) and other members of the management, who had no direct contact with horse patients (n=9). All human nasal samples were collected from both nares on one day at the beginning of the shift, before entering the clinic's operative area using nasal swabs premoistened in sterile physiological saline solution (0.85% NaCl). All volunteers participated spontaneously to the study and provided written informed consent. The sampling protocol was supervised and approved by the Committee of Science and Research Ethics, Medical Research Council, Ministry of Human Capacities, Hungary, as a protocol which does not require medical intervention (No. 10214-2/2019/EKU).

4.3.1.2 Sample processing, isolation, and identification of MRSA

Bacterial cultures of the equine samples were obtained within routine diagnostic at the Diagnostic Laboratory, Department and Clinic of Production Animal Medicine, University of Veterinary Medicine Budapest, Üllő, Hungary, on Columbia-agar plates containing 5% sheep blood (Biolab, Hungary) after 24–48 h incubation at 37 °C. Individual colonies were then subcultured under identical conditions to yield a pure culture of the bacteria. The primary identification of pure cultures consisted of Gram-staining, catalase and oxidase tests (2015). Prediction of methicillin resistance was routinely tested in case of each presumptive *Staphylococcus* sp. isolate by disc diffusion using cefoxitin disk as recommended in the standards of the Clinical and Laboratory Standards Institute (CLSI, 2009). Cefoxitin-resistant strains, producing an alpha- or both an alpha- and a beta-haemolysis on Columbia sheep blood agar were reported as haemolytic methicillin-resistant *Staphylococcus* sp. and were frozen

at -80°C. *S. aureus* were distinguished from non-*S. aureus* isolates by subsequent identification by MALDI TOF (matrix-assisted laser desorption/ionization/time-of-flight) mass spectrometry (Bruker Daltonik GmbH, Germany) at Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland. Non-*S. aureus* isolates were excluded from further investigation. Human nasal swabs underwent a previously described MRSA pre-enrichment protocol, using Muller-Hinton broth with 6.5% NaCl (3). After incubation (24 h, 37°C) a loopful (approx. 10 µL) of the pre-enrichment medium was spread onto chromogenic agar plates for selective isolation (Brilliance MRSA 2 Agar, Oxoid, Basingstoke, UK). Pure cultures of presumptive MRSA colonies were stored at -80°C until further evaluation. Cefoxitin-resistant strains of both equine and human origin were confirmed to be MRSA by MALDI TOF mass spectrometry for species identification and by the presence of the *mecA* gene by PCR (Schnellmann et al., 2006). The total number of staphylococci in the survey period was determined retrospectively, according to the case reports of the Diagnostic Laboratory, Üllő.

4.3.1.3 Antimicrobial susceptibility testing and resistance genes

Minimal inhibitory concentration (MIC) of 19 selected antibiotics were determined by microdilution in Mueller-Hinton broth using custom Sensititre susceptibility plates EUST (Thermo Fisher Scientific, Waltham, USA); MCS Diagnostics BV, Swalmen, The Netherlands) as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010). The resistance breakpoints from the CLSI document M100 for human isolates were used for clindamycin (≥ 4 mg/L), tetracycline (≥ 16 mg/L), penicillin (≥ 0.25 mg/L), chloramphenicol (≥ 32 mg/L), kanamycin (≥ 64 mg/L), quinupristin/dalfopristin (≥ 4mg/L), vancomycin (≥ 16 mg/L), gentamicin (≥ 16 mg/L), trimethoprim (≥ 16 mg/L), erythromycin (≥ 8 mg/L), ciprofloxacin (≥ 4 mg/L), cefoxitin (≥ 8 mg/L), linezolid (≥ 8 mg/L), and sulfamethoxazole (≥ 512 mg/L) as indicators of a possible acquired resistance and not for clinical purposes. For fusidic acid (> 1 mg/L) and rifampicin (> 0.5 mg/L), the resistance breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used (EUCAST 2019). For streptomycin, mupirocin and tiamulin for which no breakpoints exist, the isolates were further evaluated for the presence of a resistance mechanism if the MIC was higher than the lowest concentration measured (≤ 0.5 mg/L for tiamulin and mupirocin and ≤ 4 mg/L for streptomycin). Phenotypic resistance was confirmed by the genetic identification of the mechanism. Antibiotic resistance genes were identified using a microarray (AMR+ve-5.1 tubes; Alere Technologies GmbH; Jena, Germany) capable of detecting up to 117 different antibiotic resistance genes known to be present in Gram-positive bacteria (Perreten et al., 2005; Strauss et al., 2015). Mutations of the topoisomerase GyrA and GrlA and mutations in the RNA polymerase RpoB were investigated by amino acid analysis of translated DNA sequences obtained by PCR and Sanger sequencing (Schmitz et al., 1998; Wichelhaus et al., 1999).

4.3.1.4 Genotyping of MRSA

Genetic relatedness was confirmed using the variable numbers of tandem repeats (VNTRs) typing, *spa*-typing and multilocus sequence typing (MLST) (Francois et al., 2005; Harmsen et al., 2003; Enright et al., 2000). *Spa*-type was analysed using the Ridom StaphType software (Ridom StaphType, Ridom GmbH, Würzburg, Germany). The sequence types (ST) were determined using the Multi Locus Sequence Typing home page (http://saureus.mlst.net).

Except primary identifications of the bacteria, all further investigations were performed in the Center for Zoonoses, Animal Bacterial Diseases and Antimicrobial Resistance, University of Bern, Bern, Switzerland, during a traineeship session in September – October 2016.

4.3.2 Occurrence of equine-related MRSA strains in various epidemiological situations

4.3.2.1 Origin of samples

Hygienical screen of the Department and Clinic of Equine Medicine

After the evaluation of our first outbreak survey, we decided to investigate the occurrence and burden of this equine specific ST398-t011 clone in the Hungarian horse population. First, we assessed the MRSA contamination rate of horses leaving the DCEM (Clinic 1) and re-entering the population. Samples of 116 randomly selected outpatients were collected on the day they left the clinic between November 2017 and December 2018. Then, from June 2018, we parallelly tested the incoming patients too, as part of a general hygienical screening campaign. During this hygienical screen, 128 horses were randomly sampled upon admission, right before entering the buildings of the clinic, avoiding contamination and false positive results, a plausible risk posed by the heavy MRSA burden of the examination halls and stables, see below. Of the two groups, only 41 horses were sampled on both occasions (i.e., arriving and leaving the clinic). Samples were taken by inserting a dry, cotton tipped sterile swab (Copan, Italy) into both anterior nares around 10 cm deep and retracted while gently pushing and rotating the swab against the epithelium. Swabs were then placed into Amies transport medium supplemented with charcoal, placed to +4–8 °C and transported to the laboratory within three days.

Environmental wipes were also collected on one occasion in January 2018 from all stable units, namely the colic (n = 2), orthopaedic (n = 2) and isolation stables (n = 2); two examination halls (n = 4, two of each), and the central staff room (n = 2). We selected the sampling sites in each unit to represent both frequently used and undisturbed surfaces where dust may be absent or gather for longer periods, respectively. Since MRSA may attach and survive on dust particles (Feld et al., 2018), dust can act as a source of contamination in the hospital environment (van Duijkeren et al., 2010). Sterile, dry gauze was rubbed on an approximately 30×30 cm surface on each sampling sites, using clean, disinfected examination gloves for each sampling to avoid

cross-contamination. Samples were transferred to the laboratory and processed immediately after collection.

Countrywide sampling of equine farms without recent MRSA history

In parallel with the hygienical screen, between April 2018 and February 2019, a countrywide sampling was organized, targeting livery yards and studs (henceforth: farms) without any recent history of MRSA infection in the last six months previous to the sampling. Altogether 325 horses from 24 farms were visited and sampled, or veterinarians of the farms were asked to sample the resident horse population with the same method described in the case of the hygienical screen. Of these, samples came from 51 animals kept together with 4–10 horses, 73 with 11–20, 86 with 21–30 and 115 with ≥30 horses on the same farm. With a questionnaire, we inquired about general and animal specific factors which were previously showed to correlate with MRSA carriage: previous hospitalisation, history of former MRSA colonization and antimicrobial administration within 30 days (Weese et al., 2007). The number of veterinarians regularly visiting the farm, and the presence of other domesticated animal species living in close contact with the horses were also documented. The questionnaire served as an informed consent too, signed by the owner of the farm. In the case of those livery yards, where several owners were present, owners of the horses were informed about the sampling beforehand and could refuse to participate in the study by indicating it to the representative of the given livery, who finally signed the consent.

Targeted sampling of an equine hospital-associated outbreak

The third sampling campaign was triggered by a recent MRSA outbreak in a private veterinary clinic, in the Spring 2018. The clinic is located 80 km South from Budapest, dealing with mainly small animals and horses. Horses may also present for surgical interventions, which require intensive care and hospitalisation, like colic or orthopaedic surgery. In the recent outbreak, in May 2018 a 15-month-old mare foal developed postoperative wound infection after colic surgery. Later, it showed signs of bronchopneumonia and thrombophlebitis, and died a week later due to septicaemia in its home livery yard, after dismissed from the clinic. The foal had a history of purulent lymphadenitis and alopecia, which apparently resolved a few weeks before the recent surgery. The pathologic examination and laboratory diagnostic investigations of the carcass took place in our laboratory at Üllő, which revealed a multifocal purulent-necrotic dermatitis, cannula-associated purulent thrombophlebitis, severe embolic and purulent-necrotic bronchopneumonia. From all lesions MRSA could be isolated in almost pure cultures. We considered henceforth this horse as the index case. Meanwhile, the clinic reported further cases and asked for an on-site epidemiologic survey. Nasal swabs were collected from the hospitalized horses (n = 5) and staff members (n = 19). The environment was also screened by wiping the surgical theatre and its equipment (n = 9), stables (n = 3), the companion animal examination room (n = 3), the staff's changing room (n = 2), and the laboratory (n = 1) with the same sterile gauze technique as described in the case of DCEM.

Three farms (Farm S1-S3) epidemiologically linked to this recent outbreak were also screened for the presence of MRSA. Farm S1 was the livery from where the index case was delivered to the clinic, providing nasal swab samples from the other 71 horses, 10 staff members and let us collect 6 environmental wipes. Farm S2 received three and S3 received one horse from Clinic 2 within the same timeframe, and the bacteriological examination of postoperative wound discharge confirmed MRSA infection in the cases of three out of the four horses, affecting both farms. On Farm S2 49 horses, four staff members, and 27 environmental samples were collected, while on Farm S3 we could only sample the horses (n = 26). Horses, staff, and environment were sampled with the same techniques described above.

Targeted sampling of an apparently isolated outbreak

A fourth, apparently isolated case was presented by a stud farm reporting MRSA outbreak from February 2019 – April 2019. On 12 consecutive occasions, the farm provided a total of 322 samples from 178 horses, 59 staff members and their household members, and environmental wipes from 26 distinct places, including boxes of mares (n = 14) stables (n = 8), a transportation vehicle (n = 1), and staff rooms (n = 3). Control samples were also collected to test the efficacy of decolonisation in the case of horses (n = 18), staff members (n = 28) and hygienical measures in the mares' boxes (n = 13).

On all occasions, the targeted sampling was completed with a questionary, described above. Staff members, their household members and other related persons took part spontaneously in the sampling campaign and signed an informed written consent.

4.3.2.2 Processing of samples and phenotypic identification of MRSA strains

MRSA were cultured using a pre-enrichment method. Nasal swab samples from horses and humans were incubated overnight (16–20 h) at 37 °C in 5 ml Mueller-Hinton broth supplemented with 6.5% (w/v) NaCl. Subsequently, a loopful (about 10 µl) of the enrichment suspension was spread onto a chromogenic agar plate, selective for MRSA (BD BBL CHROMagar MRSA II, Diagon Ltd, Hungary) and incubated at 35±1 °C for 24–48 h. Positive and negative growth controls were included in each step during the protocol to monitor the performance of the pre-enrichment broth and the chromogenic agar medium. Pure cultures were then obtained by picking one medium sized, smooth, pink to mauve coloured colony per MRSA-positive plates, spread and incubated on Columbia blood agar plates for further 24 hours on 37 °C. Pure cultures of MRSA were conserved on -80 °C until further processing.

4.3.2.3 Antimicrobial susceptibility testing and resistance genes

Antimicrobial susceptibility testing (AST) and subsequent genetic investigations were performed only on the first isolates of horses, humans or sampling sites, considering the further ones as duplicates. The strains isolated from horses leaving the DCEM were excluded from AST. The AST was performed as described earlier in Section 4.2.3. The phenotypically

investigated CC398 isolates were also tested for the presence of the bifunctional aminoglycoside-modifying enzyme 6′-N-aminoglycoside acetyltransferase-2"-O-aminoglycoside phosphotransferase gene *aac(6')Ie-aph(2")Ia* by a multiplex PCR (Franz-Josef Schmitz et al., 1999). The gene confers resistance to a wide spectrum of aminoglycosides, including gentamycin, tobramycin, and kanamycin, and considered as one of the markers of equine-related CC398–t011 strains (Cuny et al., 2017). The isolates recovered from the DCEM's environmental samples were also investigated by using this multiplex PCR.

4.3.2.4 Molecular identification and genotyping of isolates

Nucleic acid was obtained from fresh pure cultures of presumptive MRSA isolates, using a direct lysis method (PrepMan® Ultra Sample Preparation Reagent, ThermoFisher, Biocenter, Hungary) and the supernatant of the lysates was stored on -20 °C. The primary identification and characterisation of MRSA was carried out by applying an array of PCRs. The first primer set (multiplex (m)PCR 1) targeted the *S. aureus*-specific *Staphylococcus* protein A gene (*spa*), the two variants of the methicillin-resistance coding genes, *mecA* and *mecC* (formerly $mecA_{LGA251}$), as well as *lukS-PV/lukF-PV* as a marker of the human-related virulence gene Panton-Valentine leukocidin (*PVL*; Stegger et al., 2012). This PCR test was applied on all isolates. Isolates originating from horses leaving the DCEM were excluded from further testing. The remaining strains were investigated by a second mPCR (mPCR 2), which amplified the *mecA* gene, served as an internal control this time, the *sau1-hsdS1*, a lineage-specific gene variant of the restriction-modification system in CC398 strains, and the human-associated *Staphylococcus* complement inhibitor gene (*scn*), marker of the phage encoded immune evasion cluster (IEC; Rasmussen et al., 2019). Typing of the *Staphylococcus* cassette chromosome carrying the methicillin resistance coding region (SCC_{mec}) was performed according to Kondo and colleagues (2007). By a further PCR test, we investigated all MRSA CC398 for the presence for the equine-specific leukocidin gene, *lukPQ* (Koop et al., 2017). According to the results of the AST and the preliminary genetic characterisations, we selected isolates of each sampling location for *spa* typing as follows: i) all isolates derived from horses, ii) representative human isolates showing the same resistance and genetic patterns as seen in horse isolates, iii) all human isolates differing from the horse isolates in terms of resistance and/or genetic characteristics, iv) and representative isolates from environmental samples, at least one per each sampling locations. *spa* typing was performed in the sequencing facility of the BIOMI Kft., Gödöllő, Hungary, according to a previously described protocol (Harmsen et al., 2003). Analysis of the sequence data and the determination of the *spa* type was carried out in the BioNumerics software 7.6 (Applied Maths NV, Belgium). In the case of the strains which were not *spa* typed, we used a mismatch amplification mutation assay (MAMA-)PCR to detect the equine lineage-specific canonical SNP 1748T (canSNP1748T) (Cuny et al., 2015b).

We considered a MRSA CC398–SCC$_{mec}$Iva strains to be equine hospital associated (EHA-) MRSA, if it was confirmed as i) gentamycin-resistant, conferred by the *aac(6')Ie-aph(2")Ia* gene and ii) were either *spa* type t011 or carried the canSNP1748T or both. Details of the implemented PCR tests are listed in Table 2.

Table 2 | *Primers and annealing temperatures of PCRs applied in the primary molecular identification and characterisation of MRSA isolates.* (*)The annealing temperature and other thermal parameters of each reaction was determined to fit the chemistry and thermocyclers used in our laboratory.

Name	Sequence	Target	Ta* (°C)	Amplicon Size (bp)	Reference
	Multiplex PCR 1 (mPCR 1)				
spa-1113f	TAAAGACGATCCTTCGGTGAGC	*spa*	58	180-600	Stegger et al., 2011
spa-1514r	CAGCAGTAGTGCCGTTTGCTT				
mecA-F	TCCAGATTACAACTTCACCAGG	*mecA*	58	162	Stegger et al., 2011
mecA-R	CCACTTCATATCTTGTAACG				
pvl-FP	GCTGGACAAAACTTCTTGGAATAT	*lukS-PV/lukF-PV*	58	85	Stegger et al., 2011
pvl-RP	GATAGGACACCAATAAATTCTGGATTG				
mecALGA251 Multi F	GAAAAAAAGGCTTAGAACGCCTC	*mecC*	58	138	Stegger et al., 2011
mecALGA251 Multi R	GAAGATCTTTTCCGTTTTCAGC				
	Multiplex PCR 2 (mPCR 2)				
CC398AF	AGGGTTTGAAGGCGAATGGG	*sau1-hsdS1*	58	296	Stegger et al., 2011
CC398r1	CAGTATAAAGAGGTGACATGACCCCT				
mecup1	GGGATCATAGCGTCATTATTC	*mecA*	58	527	Stegger et al., 2011
mecup2	AACGATTGTGACACGATAGCC				
IEC-scn-F1	TACTTGCGGGAACTTTAGCAA	*scn* (IEC)	58	130	Rasmussen et al., 2019
IEC-scn-R1	AATTCATTAGCTAACTTTTCGTTTTGA				
	canSNP1748T MAMA PCR				
1748 h1	ATGCTTTTTGGCCAGCTI**T**	canonical SNP 1748T	52	184	Cuny et al., 2015b
1748 u2	ATGCTTTTTGGCCAGCTI**G**				
1748r	ATTACTCAAGGAACTGAA				
	***lukPQ* PCR**				
lukPQ fw	CCTGATGGTGAACTGTCAGCGCAT	*lukPQ*	62	939	Koop et al., 2017
lukPQ rev	TTGTGTGCCTCGACACCCCAAC				
	resistance genes				
catpC221-F	ATTTATGCAATTATGGAAGTTG	*catp*$_{C221}$	55	435	Schnellmann et al., 2006
catpC221-R	TGAAGCATGGTAACCATCAC				
ErmC_Fw	AGTACAGAGGTGTAATTTCG	*erm(C)*	55	500	Sutcliffe et al., 1996
ErmC_Rv	AATTCCTGCATGTTTTAAGG				

4.3.3 The second retrospective investigation of *Staphylococcus* strains originating from the DCEM

Recognising the presence of Hungarian equine MRSA isolates older than the one identified in 2011 and described in the first retrospective investigations, we revised the method previously applied for isolate selection. This time, all *Staphylococcus* isolates of equine origin were considered into a second retrospective study, which were formerly not involved in the first investigations. In contrast to the previous selection pipeline, we have screened both the diagnostic and conservation records for *Staphylococcus* isolates, regardless of their described type of haemolysis or resistance characteristics. Presumptive *Staphylococcus* sp. strains were re-cultured on both Columbia blood agar and BD BBL CHROMagar MRSA II agar plates under the same conditions described in Section 4.1.2. Pure cultures were than confirmed either as *S. aureus* or MRSA by mPCR-1. MRSA isolates were further tested by a second multiplex PCR, including the primers for the CC398-specific gene fragment *sau1-hsdS1* (CC398AF, CC398r1; Stegger et al., 2012) and a primer pair specific for SCC_{mec} type IVa (Type IVa-F, 5'-GCC TTA TTC GAA GAA ACC G-3'; Type IVa-R, 5'-CTA CTC TTC TGA AAA GCG TCG-3'; Zhang et al., 2005). Considering the possible variations in the primer binding sites of the tested SCC_{mec}IVa, we performed the SCC_{mec} typing on strains producing negative results with the Zhang primer set too. A descriptive statistical analysis was performed based on the diagnostic records and newly generated molecular results. We evaluated the total number of equine samples submitted to the laboratory and every horse patient accounted for one submission record on a daily basis, regardless of the number of samples or performed bacteriological culturing. Samples submitted from the same patients on different days were treated separately. Horses were identified according to their clinical ID number and considered the same horse if tagged with the same clinical ID. If submission batch or sample yielded more than one type of *Staphylococcus* sp. (e.g., beta-haemolysing and non-haemolysing), purified strains were tested and conserved separately and identified with separate conservation numbers.

4.3.4 Molecular investigations of equine-related MRSA strains isolated between 2008-2019 in Hungary

The equine MRSA CC398 strains isolated in our workflow showed a high degree of similarity in terms of basic molecular characteristics and resistance pattern, by which they could hardly be further typed and grouped. Such subtle differences need methods with high discriminatory power, like microarray panels, PFGE or whole genome sequencing. To unravel the genetic composition and the molecular epidemiology of the Hungarian equine MRSA CC398 strains, we selected and whole genome sequenced 50 isolates. Based on the results we further characterised a larger subgroup of remaining isolates to reach a better resolution on the timescale.

4.3.4.1 Origin of strains involved in the whole genome sequencing

Isolates from the hygienical screening of the DCEM, and the targeted samplings

All t011–IVa isolates of MRSA-positive incoming horses (n = 7), and three randomly selected isolates originating from patient leaving the DCEM were chosen for whole genome sequencing. From the collection of the Clinic 2-related outbreak, one isolate per site was chosen (n = 3), while three strains from Farm S4 were selected on the same purpose. For further details see Supplementary Table S3.

Isolates from private veterinary practices

In search of equine MRSA isolates emerged outside the DCEM, we accessed and investigated the culture collection of a large-scale veterinary clinical microbiology laboratory (DUO-BAKT Labor, Veresegyház, Hungary). The laboratory receives and processes over 10,000 samples per year, originating from companion animals, horses and, in smaller numbers, farm animals in all over the country. All isolated *Staphylococcus* strains are tested for coagulase production and screened for methicillin resistance by using the cefoxitin disc diffusion method (R < 22 mm). *Staphylococcus* sp. showing beta-haemolysis, coagulase activity, and cefoxitin resistance were reposited in the culture collection between 2008–2018. Of these, 27 isolates originated from horses (n = 19) or human nasal swabs, which were epidemiologically linked to horse cases. Twenty isolates have been previously sent to the *Staphylococcus* Reference Laboratory, National Centre of Public Health, Budapest, Hungary for molecular confirmation and typing. All of these were *mecA*-positive, not-typeable (NT) by SmaI digestion and PFGE, showed the t011 *spa* type, and the SCC_{mec} was classified as type IV. The remaining seven isolates were investigated retrospectively in our laboratory with the same molecular methods, described in Section 4.2.2. and except one t034–SCC_{mec}V isolate, all typed t011–SCC_{mec}IV. Classical epidemiologic data were collected by contacting the resident veterinarians of the farms and veterinary practices and shown in Table 3. There were 16 isolates, derived from 13 horses and three humans, belonging to eight veterinary practices, other than the two equine clinics (DCEM and Clinic 2). Of these, 11 cases were in direct or indirect contact with one of the clinics. Each sampled human was in close contact with MRSA positive horses.

We selected 15 strains for WGS, based on the following criteria: i) the sample could not be found in the culture collection of the Diagnostic Laboratory, Üllő, excluding duplicates ii) the strain was cultured from a horse sample, and iii) showed the genotype t011–SCC_{mec}IV. The selected isolates are listed in Supplementary Table S3.

Table 3 | *Horse-related isolates of private veterinary practices and equine clinics.* Isolates were collected and conserved by the BUO-BAKT Veterinary Microbiological Laboratory service.

	Isolate ID	Genotype (*spa*/ SCC*mec* type)	Year	Host	Sampling site	Submitter	Clinical history and equine clinic contact
1	E03891801	t011-IV	2008	horse	synovia	Clinic 2	Sample submitted by Clinic 2
2	E03891802	t011-IV	2008	human	nasal swab	Clinic 2	Staff member of Clinic 2
3	E02721801	t011-IV	2010	horse	synovia	Clinic 2	Sample submitted by Clinic 2
4	E02721802	t011-IV	2010	horse	skin scraping	Praxis A	No data
5	E02721803	t011-IV	2010	horse	uterus	Praxis B	Mare in embryo transfer, no known equine clinic contact
6	E02721804	t011-IV	2010	horse	wound	DCEM	Hospitalization at the DCEM
7	E02721805	t011-IV	2011	horse	wound	Praxis C	Surgery at the DCEM, P.O. wound infection
8	E02721806	t011-IV	2011	horse	guttural pouch	DCEM	Hospitalization at the DCEM
9	E02721807	t011-IV	2011	horse	wound	Praxis D	Surgery at the DCEM, P.O. wound infection
10	E02721809	t011-IV	2012	horse	wound	Praxis E	Surgery, no known equine clinic contact
11	E02721810	t011-IV	2013	human	nasal swab	Praxis E	MRSA positive equine contact
12	E02721812	t011-IV	2013	human	nasal swab	Praxis E	MRSA positive equine contact
13	E02721813	t011-IV	2013	human	nasal swab	Praxis E	MRSA positive equine contact
14	E02721814	t011-IV	2013	human	nasal swab	Clinic 2	Staff member of Clinic 2
15	E02721815	t011-IV	2013	human	nasal swab	Clinic 2	Staff member of Clinic 2
16	E02721816	t011-IV	2013	horse	wound	Clinic 2	Sample submitted by Clinic 2
17	E02721817	t011-IV	2013	horse	wound	Praxis E	No known equine clinic contact
18	E02721819	t011-IV	2013	horse	wound	Clinic 2	Sample submitted by Clinic 2
19	E03891803	t011-IV	2013	human	nasal swab	Clinic 2	Staff member of Clinic 2
20	E02721820	t011-IV	2013	human	nasal swab	Clinic 2	Staff member of Clinic 2
21	E02721829	t011-IV	2016	horse	wound	Praxis F	Previous hospitalization at the DCEM
22	E03891804	t011-IV	2016	horse	fistula	Praxis D	P.O. wound infection, previous hospitalization at the DCEM
23	E03891805	t011-IV	2017	horse	skin scraping	Praxis G	Contact with DCEM outpatients
24	E03891806	t011-IV	2017	horse	skin scraping	Praxis E	Previous emergency treatment at the DCEM
25	E02791800	t011-IV	2018	horse	wound	Praxis A	Previous hospitalization in Clinic 2
26	E03891807	t011-IV	2018	horse	wound	Praxis A	Previous hospitalization in Clinic 2
27	E03891808	t034-V	2018	horse	trachea	Praxis H	No data

Isolates from the Diagnostic Centre and the Department and Clinic of Equine Medicine, Üllő

Non-duplicate CC398–SCC$_{mec}$IVa MRSA strains isolated from the samples of the DCEM between 2009–2017 were selected based on the following criteria: i) to be able to analyse at least two strains per each year, ii) which were isolated at least three months apart iii) from clinical lesions of distinct hospitalized horses at the DCEM. From 2018 two strains originating from leaving horses were selected, see Part A) of this section. The two horses tested MRSA-negative upon admission, since their consecutive positive sample were considered as of nosocomial origin (E00021807 and E03761803), and the isolates were confirmed by *spa* and SCC$_{mec}$ typing before submitting for WGS. In 2009, where only three isolates were conserved, of which two were from the same horse. Only one of the duplicate isolates was submitted for WGS.

4.3.4.2 Whole genome sequencing, genomic analysis, and supplementary molecular investigations

Isolation of DNA, library preparation, and sequencing

Genomic DNA was isolated from pure culture of MRSA using the NucleoSpin Microbial DNA Kit (Macherey-Nagel) according to the manufacturer's instructions. The quantity and quality of DNA was assessed by measurements using a Qubit 4.0 fluorometer (Invitrogen, Waltham, USA) and Tapestation 4150 systems (Agilent, Santa Clara, USA). The NGS libraries were prepared using the Nextera DNA Flex Library Prep Kit (Illumina, Eindhoven, The Netherlands) with Nextera DNA CD Indexes. The NGS libraries were sequenced on an Illumina MiSeq instrument using the MiSeq Reagent Kit v2 or v3 obtaining paired end 150 bp, 250 bp or 300 bp reads, depending on the flow cell size and WGS run, respectively.

Bioinformatic analysis

All bioinformatic investigations, including quality control of raw reads, de-novo assembly of contigs, cgMLST analysis, resistance and virulence gene mapping was performed as outlined in Section 4.1.4, with some additional steps. In the case of the Hungarian isolates, we also generated an allele matrix by involving those whole genome MLST loci which were present in each investigated isolate. This maximum common genome (mcg)MLST scheme contained 2424 loci, a subset of an identified total of 2643. Strains were also tested for the presence of the canonical SNP canSNP1748T by performing an in-silico MAMA PCR with primers 1748 h1 and 1748r. The number of allowed mismatches was set to zero and only PCR products of the expected size were accepted. Genetic vicinity of certain virulence and resistance genes were visualised and further investigated in Geneious Prime.

Supplementary molecular investigations of the equine hospital-associated MRSA CC398 culture collection originating from the DCEM

We chose and tested for genetic markers, that were likely indicating microevolutionary changes in the EHA-MRSA lineage. Resistance genes *cat*$_{pC221}$ and *erm*(C) and virulence gene *lukPQ* were investigated using primers and PCR conditions as formerly described in this section, see Table 2. For the testing of *qacJ* gene conferring resistance to quaternary ammonium compounds, we designed a new set of primers based on our whole genome sequence data using the Primer3 plugin of Geneious Prime software. Primers qacJ_Fw1 (5'-TAT GCA ACT TGG GCA GGG TT-3') and qacJ_Rev1 (5'-ACG TTA AGA AGC ACA ACA CCA A-3') were selected to yield a 135 bp amplicon, which is also appropriate for real-time PCR chemistry using intercalating dyes, like SYBR Green or EVA Green. For routine testing, positive and negative controls were selected from the culture collection according to the isolates' WGS-based in-silico PCR results. The phenotypic rifampicin resistance of the strains was investigated either by collecting the respective data from the case reports or isolates were re-cultured and subsequently tested using the disc-diffusion method, if the susceptibility testing was previously not performed.

To put the degree of genetic relatedness of the Hungarian equine MRSA strains into a wider context, we compared our strains to other European CC398-SCC$_{mec}$IV MRSA isolates originating eighter from horses or humans dealing with horses. Whole genome sequence data of previously sequenced isolates published by others or identified by the Similar Genome Finder service of PATRIC 3.6.3 (Wattam et al., 2017) were investigated by the same cgMLST scheme, as previously described in this section, with a slight modification. Data were analysed only by using the assembly-based method, as some isolates were only available as assembled draft genomes (isolates 111-135, Supplementary Table S3). Virulence and resistance genes were also investigated as previously described. Further details of the involved European strains are also summarized in Supplementary Table S3.

5. Results

5.1 Occurrence rates and genomic characteristics of pig-related MRSA strains

5.1.1 Number of MRSA-positive farms and MRSA in swine professionals

A total of 40 holdings participated in the survey, including 38 farms rearing sows and two fattening farms, representing all major pig-producing regions of the country (Figure 1). The number of sows per breeding farm varied from 470 to 3000, with the total number accounting for 13% of the registered sows in Hungary in 2019 (personal communication of the National Food Chain Safety Office, Budapest, Hungary). Both fattening farms dealt with approximately 2000 fatteners per fattening cycle. Of the 40 sampled farms, 33 were MRSA positive (82.5%) during the recent sampling, and the proportion of the positive breeding and production farms was 31/38 (81.6%). Interestingly, only a quarter of the farms (10/40) reported a previous laboratory confirmation of MRSA. All but one previously MRSA-positive farm tested positive again. In the case of four farm samples, there were two distinct phenotypes present on the chromogenic agar plate – and both were selected for further testing – resulting in 37 farm isolates.

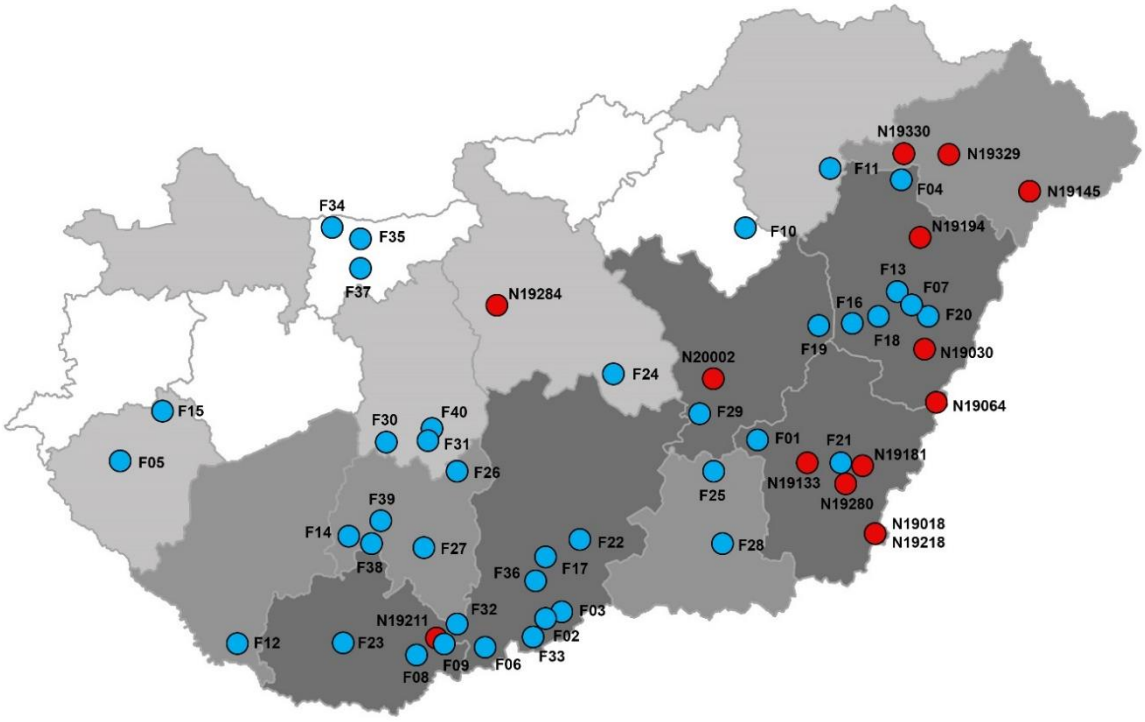

Figure 1 | *Location of farms and geographical origin of human clinical LA-MRSA isolates involved in the study.* Blue circle, swine farms; red circle, origin of human clinical MRSA isolates. Farms and human isolates are numbered as referred in the text. Counties shaded according to the number of sows in year 2019: 20,000-32,000 (dark grey); 10,000-20,000 (middle grey); 5,000-10,000 (light grey); 1,000-5,000 (white)

Out of the 36 swine professionals in contact with the farms, 27 provided sample, of whom 25 were veterinarians and two were farm managers. Nineteen of them were tested positive for MRSA (70.4%), including both farm managers. In a single case, two phenotypically different isolates were further investigated from the same sample (Farm 28); thus, there were a total of 20 swine professional isolates. The number of MRSA-positive farm–swine professional couples was 14. In only two cases, both the farm and the contact person were negative. In six cases the farms were tested positive, while the swine professionals were tested negative, and in five cases the farm was negative but the human sample was tested positive.

Of note, none of the MRSA-negative samples yielded *S. aureus* on the *S. aureus*-selective chromogenic agar medium cultured in parallel, while other staphylococci grew on most plates, ruling out failure of sampling or culturing in these cases (data not shown).

5.1.2 Molecular epidemiology, virulence, antimicrobial susceptibility, and resistance genes of swine farm-related MRSA isolates

Among the 57 MRSA isolates chosen for further testing, all but a sequence type 45 (ST45) isolate belonged to MLST CC398, of which most represented the founder ST, ST398 (52/56). Three isolates were ST541, a single locus variant (SLV) of ST398, and a new SLV was also identified in the case of the isolate F19_E1, assigned to ST6268. An array of *spa* types related to CC398 were identified, with t034 (n = 22; 38.6%) and t011 (n = 21; 36.8%) being the two most prevalent variants. A few other isolates carried *spa* types t4208 (n = 3) and t1197 (n = 2), and one each represented *spa* types t571, t1250, t1255, t1451, and t4571. A new, yet unassigned *spa* type was identified among three further isolates by the WGS-based analysis, all originating from the same farm. The majority of the isolates carried the Vc (5C2&5) variant of the *Staphylococcus* cassette chromosome *mec* (n = 55), and a single isolate had type IVb (2B). A swine professional isolate was *scn*-positive, carried SCC_{mec} type IVa, identified as *spa* type t330, and belonged to a human-associated ST45. This isolate was considered not to be livestock-associated and was excluded from subsequent analyses.

The human clinical isolates (n = 14) all belonged to CC398, and except for three isolates, they were all typed as ST398. Two isolates (N19145 and N19284) belonged to ST1232 and one isolate (N19018) was an SLV of ST398 and assigned to a new ST, ST8001. Six isolates were *spa* type t011, five were t034, two were t4208, and one was t3275. Except for two, all isolates belonged to the Vc (5C2&5) SCC_{mec} type. The two ST1232 isolates lacked a second ccrC1 allele and thus were typed as V (5C2). The major characteristics of the isolates that underwent WGS are summarised in Supplementary Table S2.

Phenotypic antimicrobial susceptibility and resistance genes of farm-related isolates

All analysed farm-related LA-MRSA isolates (n = 56) were susceptible to rifampicin, vancomycin, mupirocin, and sulfamethoxazole, while resistance to fusidic acid or linezolid was observed only in single isolates (1.8%) (Table 4). In addition to resistance to the beta-lactam

antibiotics penicillin and cefoxitin, all isolates were also resistant to tetracycline. It should be noted that three isolates were apparently susceptible to cefoxitin (MIC = 4 mg/L) but considered to be MRSA as these strains had previously grown on selective medium containing cefoxitin and tested positive for the *mecA* gene by PCR. There was higher resistance in the case of clindamycin (83.9%), trimethoprim (57.1%), and quinupristin/dalfopristin (48.2%). Almost three quarter of the isolates showed a non-wild-type phenotype when tested with tiamulin. Half of the isolates were resistant or expressed a non-wild-type phenotype to at least six tested antibiotics other than beta-lactams.

Table 4 | *Phenotypic antimicrobial susceptibility of 56 swine-related livestock-associated methicillin-resistant Staphylococcus aureus isolates.*

		Farm environment isolates (n = 37)			Swine professional isolates (n = 19)			Total (n = 56)		
		R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Penicillin		37 (100)	-	0 (0)	19 (100)	-	0 (0)	56 (100)	-	0 (0)
Cefoxitin		36 (97.3)	-	1 (2.7)	17 (89.5)	-	2 (10.5)	53 (94.6)	-	3 (5.4)
Tetracycline		37 (100)	0 (0)	0 (0)	19 (100)	0 (0)	0 (0)	56 (100)	0 (0)	0 (0)
Clindamycin		29 (78.4)	0 (0)	8 (21.6)	18 (94.7)	1 (5.3)	0 (0)	47 (83.9)	1 (1.8)	8 (14.3)
Trimethoprim		21 (56.8)	-	16 (43.2)	11 (57.9)	-	8 (42.1)	32 (57.1)	-	24 (42.9)
Quinupristin/dalfopristin		20 (54.1)	4 (10.8)	13 (35.1)	7 (36.8)	8 (42.1)	4 (21.1)	27 (48.2)	12 (21.4)	17 (30.4)
Ciprofloxacin†		15 (40.5)	3 (8.1)	-	10 (52.6)	5 (26.3)	-	25 (44.6)	8 (14.3)	-
Chloramphenicol		17 (45.9)	0 (0)	20 (54.1)	7 (36.8)	0 (0)	12 (63.2)	24 (42.9)	0 (0)	32 (57.1)
Erythromycin		15 (40.5)	0 (0)	22 (59.5)	8 (42.1)	0 (0)	11 (57.9)	23 (41.1)	0 (0)	33 (58.9)
Gentamicin		4 (10.8)	-	33 (89.2)	3 (15.8)	-	16 (84.2)	7 (12.5)	-	49 (87.5)
Fusidic acid		1 (2.7)	-	36 (97.3)	0 (0)	-	19 (100)	1 (1.8)	-	55 (98.2)
Linezolid		1 (2.7)	-	36 (97.3)	0 (0)	-	19 (100)	1 (1.8)	-	55 (98.2)
Rifampicin		0 (0)	0 (0)	37 (100)	0 (0)	0 (0)	19 (100)	0 (0)	0 (0)	56 (100)
Vancomycin		0 (0)	-	37 (100)	0 (0)	-	19 (100)	0 (0)	-	56 (100)
Sulfamethoxazole‡		0 (0)	-	37 (100)	0 (0)	-	19 (100)	0 (0)	-	56 (100)
	ECOFF (mg/L)	NWT (%)		WT (%)	NWT (%)		WT (%)	NWT (%)		WT (%)
Tiamulin§	2	25 (67.6)		12 (32.4)	16 (84.2)		3 (15.8)	41 (73.2)		15 (26.8)
Streptomycin§	16	9 (24.3)		28 (75.7)	5 (26.3)		14 (73.7)	14 (25.0)		42 (75.0)
Kanamycin§	8	4 (10.8)		33 (89.2)	3 (15.8)		16 (84.2)	7 (12.5)		49 (87.5)
Mupirocin§	1	0 (0)		37 (100)	0 (0)		19 (100)	0 (0)		56 (100)

R, resistant; I, susceptible, increased exposure; S, susceptible. †The susceptibility of 23 isolates could not be determined, because their minimal inhibitory concentration (MIC) fell below the lowest concentration measured (0.25 mg/L). ‡MIC values evaluated according to the Clinical and Laboratory Standards Institute (CLSI) criteria. §Epidemiological cut-off (ECOFF) values were used to determine wild-type (WT) and non-wild-type isolates.

Twenty-four resistance genes and three non-synonymous point mutations in two genes conferring resistance to antibiotics were identified, in good agreement with phenotypic data (Table 5). However, there were also discrepancies between the phenotypic and genotypic results in several isolates. Eight isolates showed resistance towards chloramphenicol, without an identifiable underlying genetic trait. In addition, 14 isolates were apparently resistant to the quinupristin/dalfopristin combination (≥ 4 mg/L); however, each of them carried only the *lsa*(E) gene conferring resistance to streptogramin A antibiotics.

Table 5 | *Antimicrobial resistance determinants in 56 swine-related livestock-associated methicillin-resistant Staphylococcus aureus isolates.* Resistance genes and mutations are listed as identified using ResFinder. The occurrence rates are indicated in parentheses. Genes with bold characters are multi-drug resistance genes – that is, they code resistance to at least three antimicrobial classes.

Antimicrobial class	**Resistance genes (%)**
Beta-lactams	*blaZ* (100%); *mecA* (100%)
Tetracyclines	*tet*(M) (98.2%); *tet*(K) (96.4%); *tet*(L) (5.4%)
Phenicols	*fexA* (28.6%)
Aminoglycosides	*aac(6')-aph(2'')* (12.5%); *aadD* (5.4%); *ant(9)-Ia* (50.0%)
Streptomycin	*ant(6)-Ia* (5.4%); *str* (19.6%)
Trimethoprim	*dfrG* (44.6%); *dfrK* (12.5%)
Fusidic acid	*fus*(C) (1.8%)
Lincosamides	*lnu*(A) (5.4%); *lnu*(B) (48.2%)
Lincosamides, pleuromutilins, streptogramin A	***lsa*(E)** (48.2%); ***vga*(A)$_{LC}$** (10.7%); ***vga*(A)$_{V}$** (8.9%); ***vga*(E)** (3.6%)
Macrolides, lincosamides, streptogramin B	***erm*(A)** (5.4%); ***erm*(B)** (10.7%); ***erm*(C)** (26.8%)
All phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A	***cfr*** (1.8%)
	Gene mutations and corresponding amino acid change
Fluoroquinolones	*gyrA* [S84L] (33.9%)
	grlA [S80F] or *grlA* [S80Y] (48.2%)

Relatedness of MRSA CC398 isolates

The allelic differences among the 70 compared isolates ranged 0 to 189.8 in almost all cases based on the cgMLST analysis (Supporting Information S4). There were two pairwise comparisons, however, where the differences were > 200 and not displayed by the BioNumerics software. These were the human-derived clinical isolates of ST1232, which were distantly related to all others (minimum allelic difference > 100) and thus were considered outliers (N19145 and N19284). On the other hand, they were rather closely related and showed a 5.0 allelic difference. The groups of farm-environmental and swine professional isolates showed a comparable yet moderate within-group heterogeneity, with allelic differences ranging from 0 to 125.9 (median 81.5) and from 0 to 123.0 (median 81.2), respectively. Except for the two outliers, the human clinical isolates were more distantly related, differing by a minimum of 29.3 alleles (maximum: 189.8, median: 144.6).

Both UPGMA trees, with (Supporting Information S5) or without (Figure 2) the Danish isolates, were split into two main groups, Groups 1 and 2. The groups comprised clusters of closely related isolates (Clusters C1 to C5), identified based on the trees and by applying the threshold of ≤ 24 allelic differences.

The SNP analysis identified 26–946 pairwise SNP differences (median: 241) in the case of all 79 LA-MRSA isolates. Within-group heterogeneity was indicated by 41–485 pairwise SNP differences (median: 239) among the farm environmental isolates, and the swine professional strains differed by 44–281 SNPs (median: 220). The human clinical isolates showed 37–335 SNP differences (median: 236) within the group. The maximum likelihood phylogeny tree reflected most characteristics of the UPGMA trees, as the previously identified clusters C1–C3 were well recognisable, while the genetic heterogeneity of C4 and C5 became more apparent (Figure 3).

Evaluating the relatedness of swine–professional, farm–environmental, and human clinical isolates

All whole genome–sequenced swine professional LA-MRSA isolates (n = 19) showed close relatedness (≤ 24.0 allelic difference) to at least one environmental isolate. When considering the 13 cases where both the farm and the swine professional tested positive for LA-MRSA, there were almost identical pairs. In eight cases, the allelic difference between the environmental and swine professional isolates was between 0 and 4.5, and the pairwise SNP differences was between 26 and 73. From the remaining five environments, three nasal swab isolate pairs showed 114.4–122.5 allelic differences or 230–248 SNPs. In one case (Farm 8), there were two phenotypically distinct environmental strains isolated, of which one did not differ from the nasal swab isolate (F08_E1; 0 allelic difference and 73 SNPs), while the other was rather distantly related (F08_E2; > 100 allelic difference and > 247 SNPs). All swine professionals who carried other genotypes than those detected on the farms were veterinarians. Only one of them reported no professional contact with other animals; the rest either worked with other farm animal species, mainly ruminants (n = 1), or also with swine on other farms (n = 2).

Nine out of 14 human clinical isolates clustered together with at least one farm environmental isolate, which was considered to be closely related based on the cgMLST analysis (Figure 2). Two of them also showed < 5 allelic differences with farm isolates (N19211 and N20002). Of note, isolates N19211 and F09_E1 (2 allelic differences or 52 SNPs) both exhibited the phenicol-lincosamide-oxazolidinone-pleuromutilin-streptogramin A ($PhLOPS_A$) multi-resistance pattern. The resistance could be attributed to the cfr gene, encoded on a large pSA737-like plasmid 40 kB in size.

Four isolates carried the immune evasion cluster (IEC) gene cluster, but only one of them showed a minimum of 15 pairwise allelic differences or ≥ 56 SNPs compared with farm environmental isolates; the rest were separated by a minimum of 48.4 allelic differences or 105 SNPs. Two of them were the ST1232 outliers, isolates N19145 and N19284. Only these two isolates encoded the Panton-Valentine leukocidin genes (*lukS-PV/lukF-PV*).

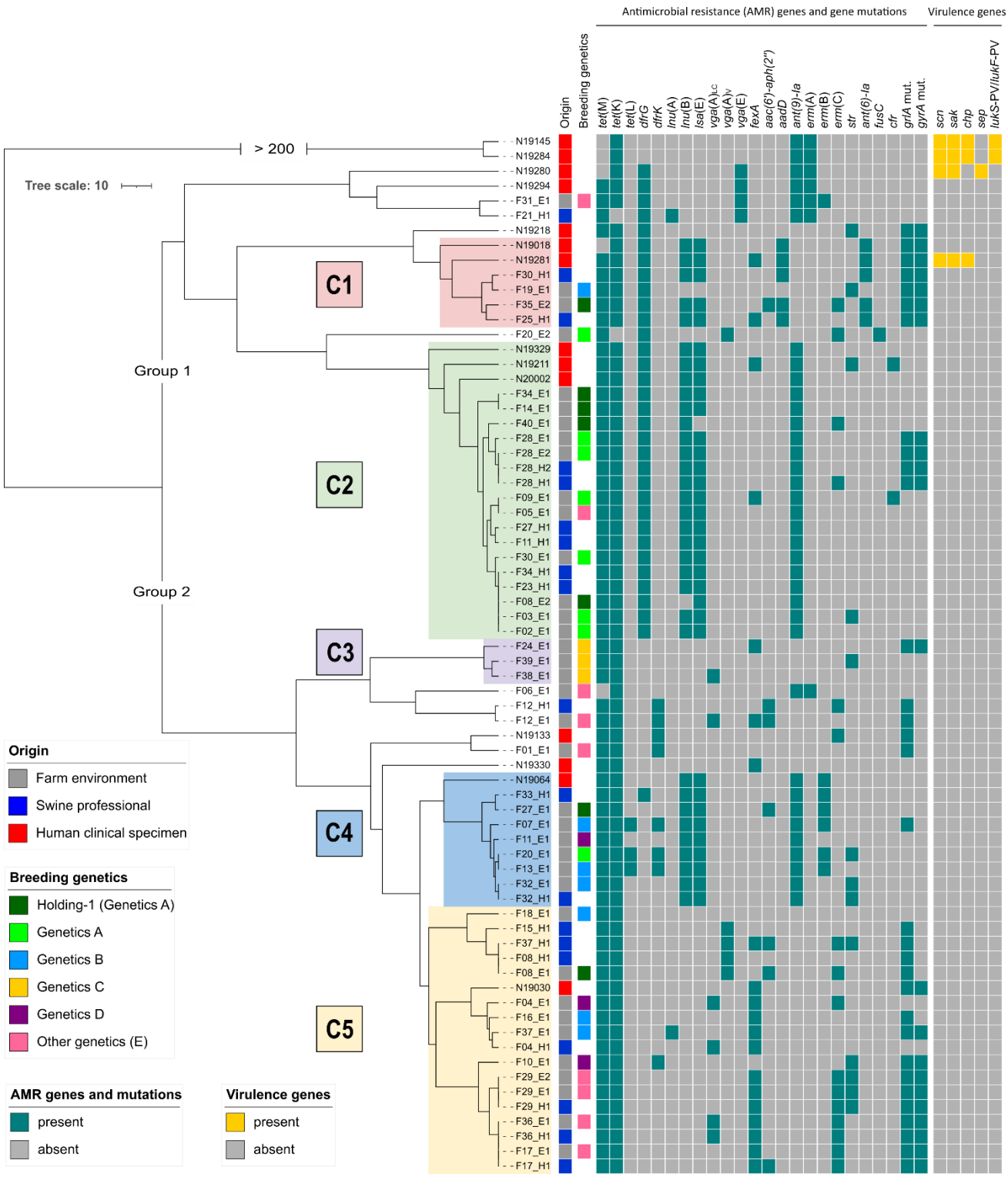

Figure 2 | *Relatedness and genetic traits of livestock-associated methicillin-resistant Staphylococcus aureus (MRSA) clonal complex (CC) 398 strains isolated from farm environments, swine professionals, and human clinical specimens.* The unrooted unweighted pair group method with arithmetic mean (UPGMA) tree was generated using the core genome multilocus sequence typing (cgMLST) distance matrix of 70 MRSA isolates. The identified genetic clusters (C1–C5) are highlighted. The tree scale bar indicates a 10 allelic difference. The branch showing an allelic difference > 200 was trimmed automatically by the BioNumerics software. Please note the strong association between the farm genetics and the clustering of isolates in C2 and C3.

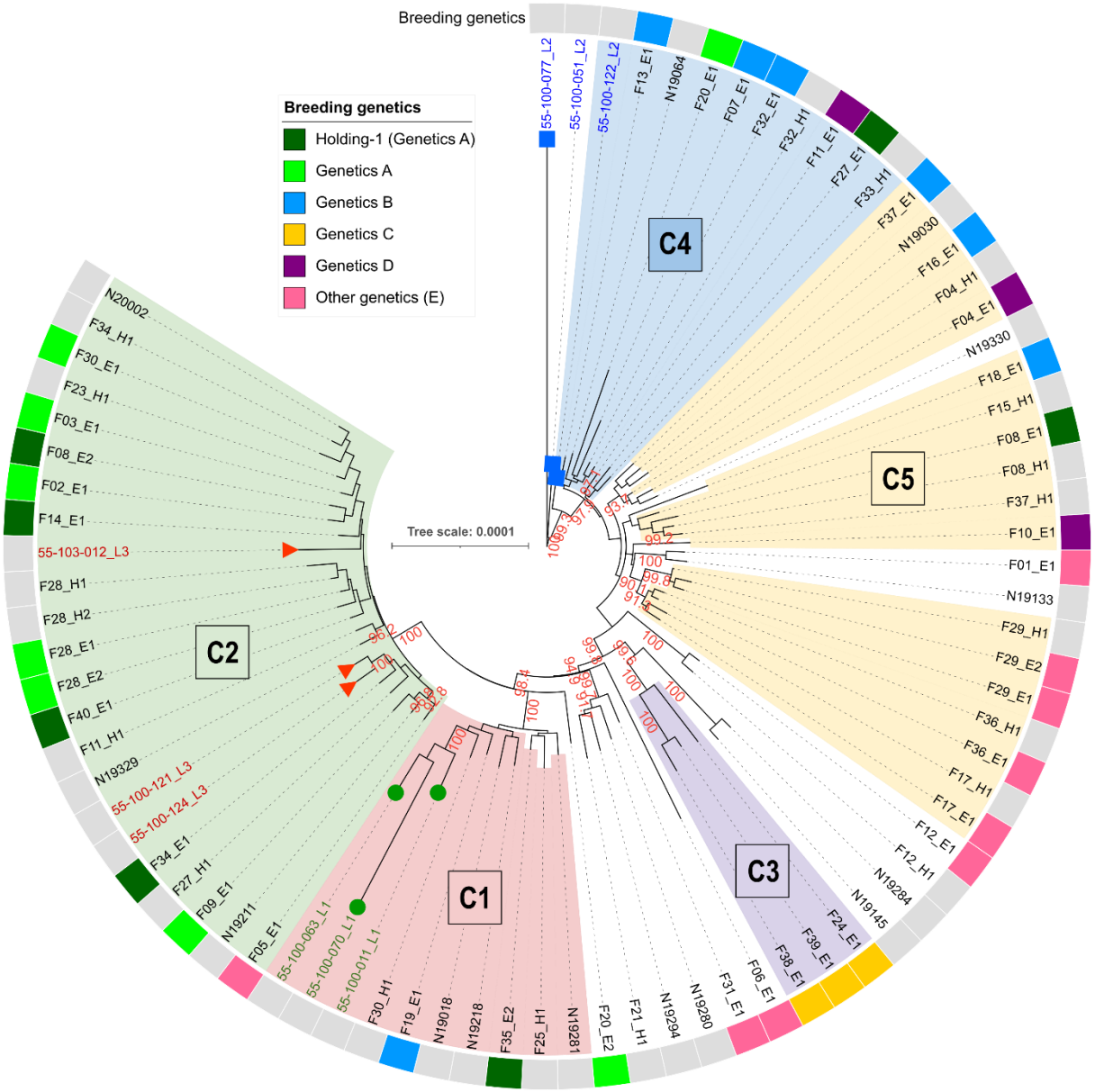

Figure 3 | *Maximum likelihood phylogeny of Hungarian and Danish livestock-associated methicillin-resistant Staphylococcus aureus (LA-MRSA) isolates*. The colours indicate the clusters identified by core genome multilocus sequence typing (cgMLST) analysis. The scale bar indicates substitutions per site; bootstrap values are shown if > 90 (red letters). There is a clear clustering of Danish L3 isolates (red triangle) with the Hungarian C2 cluster. The L1 isolates (green circle) are clustered with C1 isolates, but rather distantly related. L2 isolates (blue square) are more distantly related to the Hungarian LA-MRSA strains.

Farms breeding genetics and relatedness of MRSA isolates

In some cases, there was a strong association between the farms' breeding genetics and the genetic clustering of corresponding isolates. Farms could be classified into four well-defined groups (A–D) according to their breeders' genetics, while Genetics E comprised all other farms with mainly highly mixed breeds. Purchase networks of holdings were also identified. Holding-1 is a large integration with two nucleus farms, which has imported breeding Genetics A exclusively from Denmark in years prior to the recent study. These two nucleus farms, including Farm 14, supplied the multiplier and production farms of the integration (n = 5) and farms of other holdings (n = 6) (Figure 4). Most farms of Genetics A (10/12) clustered in Group 1, and mainly within cluster 2, according to the cgMLST analysis.

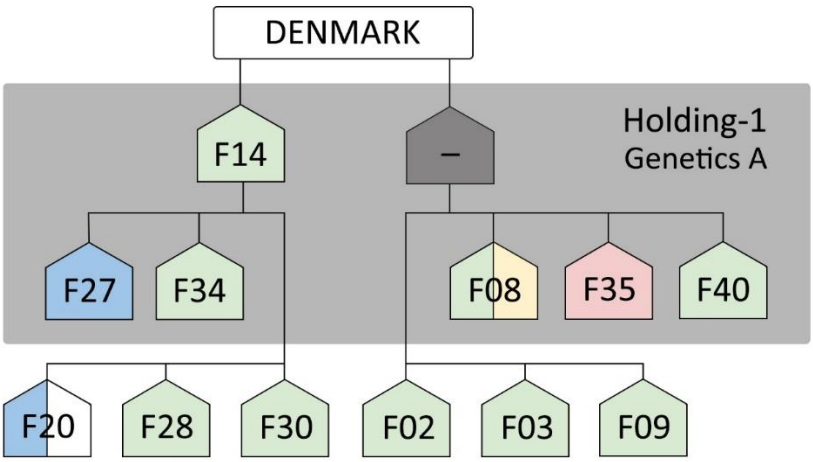

Figure 4 | *Overview of the purchase network of Holding-1 rearing breeding Genetics A.* The farm shading corresponds to the methicillin-resistant *Staphylococcus aureus* clusters identified by core genome multilocus sequence typing (cgMLST) analysis: C1 (red), C2 (green), C4 (blue), C5 (yellow) and no identified genetic cluster (white).

All but one farm breeding Genetics B were clustered in C4 (n = 3) and C5 (n = 3) (Figures 2 and 3). The farms of Genetics C had purchased their animals from the same source and formed the well-separated cluster C3 in Group 2. The three farms rearing Genetics D were scattered among C4 (n = 1) and C5 (n = 2), rather distantly related (pairwise allelic difference > 17.5). Among the farms of different mixed genetics, there were more in each main genetic group (Groups 1 and 2) of the cgMLST similarity trees. However, Farms 17, 29, and 36 formed a well-separated sub-cluster within C5 (pairwise allelic difference < 3.1), and two of these farms were known to supply each other with animals (Figures 2 and 3).

Relatedness of Hungarian and Danish LA-MRSA isolates

According to the cgMLST tree, the randomly selected Danish isolates of Lineages 1 and 3 clustered together with the Hungarian MRSA strains (Supporting Information S5). The three L1 isolates showed 10.1–27.2 allelic differences from the C1 strains, and similarly, L3 isolates showed 5.1–34.3 allelic differences from C2 isolates. Despite no obvious clustering, the L2 isolates were rather close to the Hungarian C4 strains, showing 8.3–45.5 (median: 17.5) pairwise allelic differences. The SNP analysis reinforced only the close relatedness of all three L3 to C2 isolates, while only two L1 isolates and a single L2 isolate fell close to the C1 and C4 strains on the phylogenetic tree, respectively (Figure 3).

5.2 Occurrence and characterisation of methicillin-resistant *Staphylococcus aureus* isolated from bovine milk

5.2.1 Occurrence rates and genotypes

Between 2003 and 2018, 27 MRSA strains originating from 10 dairy farms were deposited in the culture collection of the Veterinary Diagnostic Directorate. From these, 12 were recovered from one farm and were partly characterised by Juhász-Kaszanyitzky et al. (2007) (Figure 5). The ST1–t127–SCC$_{mec}$IV genotype occurred on two further unrelated farms (one and seven isolates, respectively), also causing subclinical mastitis outbreaks. Variable genotypes were identified sporadically from other farms: ST22–t032–SCC$_{mec}$IV (n = 3) from three farms; a newly described double locus variant of ST97, ST5982–t458–SCC$_{mec}$IV (n = 2) from two farms; and the ST398–t011–SCC$_{mec}$IV (n = 1) and V (n = 1) from two respective farms. The prospective screening resulted in 626 individual SA isolates originating from 42 dairy farms (1–117 isolates per farm). Among these, only four MRSA strains (0.48 %) were identified. Most strains (591/626) arrived from farms with five or more SA isolates during the survey period (20/42 farms). The four MRSA strains were recovered from three farms (7.14 %) and all of them belonged to the clonal complex 398. Among these, *spa*-type t19251 is described here for the first time. All investigated MRSA strains proved to be PVL-negative (Table 6).

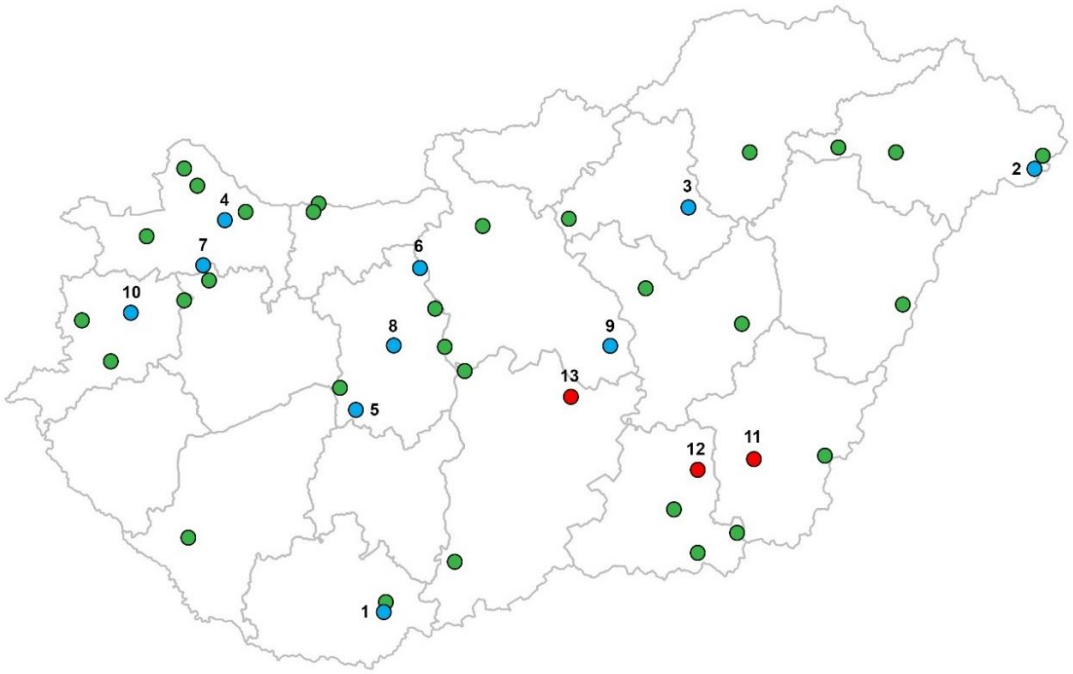

Figure 5 | *Location of dairy farms involved in the study.* The MRSA status of farms is colour coded: blue circle, origin of MRSA strains of the retrospective investigations; red circle, origin of MRSA strains from the passive surveillance; green circle, farms without MRSA in the passive surveillance. MRSA positive farms are numbered as referred in Table 6.

5.2.2 Antimicrobial susceptibility

All but two of the 14 representative isolates were resistant to at least four antimicrobial classes, among which resistance to tetracycline (11/14), clindamycin (10/14), gentamycin (10/14), ciprofloxacin (9/14) and streptomycin (8/14) were the most prevalent. All investigated isolates were susceptible to quinupristin-dalfopristin and tigecycline, and only sporadic resistance (n = 1) could be observed in the case of the following compounds: chloramphenicol, fusidic acid, linezolid, and rifampicin. The molecular investigation of the tetracycline and aminoglycoside resistance genes corresponded well with the phenotype of the strains: in all but one strain at least one gene encoding the respective resistance could be detected. Further details of the results are summarised in Table 6.

Table 6 | *Methicillin-resistant* Staphylococcus aureus *strains of milk origin.* CHL, chloramphenicol; CN, gentamicin; CIP, ciprofloxacin; DA, clindamycin; E, erythromycin; FA, fusidic acid; K, kanamycin; LNZ, linezolid; P, penicillin; SYN, quinupristin-dalfopristin; RA, rifampicin; S, streptomycin; SXT, trimethoprim and sulfamethoxazole; TE, tetracycline; TIA, tiamulin, TGC, tigecycline; TOB, tobramycin; *aacA-aphD*, bifunctional aminoglycoside acetyltransferase and phospotransferase (AAC/APH) gene; aphA3, aminoglycoside O-phosphotransferase APH(3')-IIIa gene; *aadD*, aminoglycoside adenyltransferase AadD gene; *aadE*, aminoglycoside adenyltransferase AadE gene; *tet*(K), tetracycline resistance protein TetK gene; *tet*(M), tetracycline resistance protein TetM gene; MLST, multilocus sequence type; SA, *Staphylococcus aureus*; *spa*, *Staphylococcus aureus* protein A sequence type, SCC*mec*, *Staphylococcus* Cassette Chromosome *mec* type; N/A, not applicable; ND, not determined.

Number of Farm	Laboratory	Year of Isolation	Number of MRSA Isolates	SA/Total Milk Sample in Survey	Genotype (MLST-*spa*-SCC$_{mec}$)	Resistance	Intermediate Resistance
STRAINS OF THE RETROSPECTIVE SURVEY							
1.	VDD	2003	1	N/A	ST1-t127-IV	CN, K, TOB [*aacA-aphD*, *aphA3*]; DA; E; P; S [*aadE*]; TE [*tet*(K)];	RA
2.	VDD	2004	12	N/A	ST1-t127-IV	DA, E, K [*aphA3*], P, S [*aadE*], TE [*tet*(K)]	RA
3.	VDD	2011	1	N/A	ST5982-t458-IV (CC97)	CN, K, TOB [*aacA-aphD*, *aphA3*] DA; E; P; S [*aadE*], STX; TE [*tet*(L)]; TIA	SYN
4.	VDD	2011	1	N/A	ST5982-t458-IV (CC97)	CIP; CN, K, TOB [*aacA-aphD*, *aphA3*] DA; P; S [*aadE*]; STX; TE [*tet*(L)]; TIA	SYN
5.	VDD	2013	1	N/A	ST398-t011-IV	CIP, CN, K, TOB [*aacA-aphD*, *aphA3*]; P; STX; TE [*tet*(M)]	RA
6.	VDD	2014	1	N/A	ST22-t032-IV	CIP, P	SYN
7.	VDD	2015	1	N/A	ST22-t032-IV	CIP, P	-
8.	VDD	2015	1	N/A	ST22-t032-IV	CIP; K [ND]; LNZ; P	-
9.	VDD	2018	7	N/A	ST1-t127-IV	CIP; DA; E; K [*aphA3*]; P; S [*aadE*]; TE [*tet*(K)]	-
10.	VDD	2018	1	N/A	ST398-t011-V	DA, P, TE [*tet*(K), *tet*(M)], TIA	SYN
STRAINS OF THE PASSIVE SURVEILLANCE							
11.	UVMB	2017	1	26/287	ST398-t19251-V	CIP, DA, E; K [*aphA3*]; P, S [*aadE*], TE [*tet*(K), *tet*(M)], TIA	SYN
	UVMB	2018	1		ST398-t011-V	CHL; CIP; DA; K [*aphA3*]; P; RA; S [ND]; SXT; TE [*tet*(K)], TIA	SYN
12.	UVMB	2018	1	1/64	ST398-t034-V	DA, E; K, TOB [*aadD*]; FA, P, SXT, TE [*tet*(K), *tet*(M)], TIA	SYN
13.	ÁT Kft	2018	1	2/2	ST398-t1344-V	CIP, DA, E, P, S [*aadE*], TE [*tet*(K), *tet*(M)], TIA	SYN

5.3 Investigating MRSA strains isolated from horses and related humans in Hungary

5.3.1 Characterisation of MRSA strains isolated in the Department and Clinic of Equine Medicine between 2011 and 2016

Forty MRSA strains were identified from 43 cefoxitin resistant, haemolytic *Staphylococcus* sp. strains between July 2011 and May 2016 at the DCEM. The MRSA strains originated from different skin and soft tissue infection sites or from routine nasal swabs and tracheal wash samples of 36 equine patients. The three cefoxitin-resistant non-*S. aureus* strains consisted of two *S. haemolyticus* and one *S. sciuri*-like strains, each isolated from nasal swab samples of two horses. These latter three isolates were excluded from further evaluation. The isolation frequency and main characteristics of the total of 87 horse-related staphylococci, obtained during the survey period are presented in Figure 6. Additional ten MRSA strains were isolated from nasal swabs of 36 clinic staff members in October 2017. From these staff members two were veterinary internists, six were technicians and two were from the management of the DCEM.

All equine MRSA isolates belonged to ST398, *spa* type t011 and contained the same SCC$_{mec}$IV element. In addition, all of them exhibited the same VNTR profile, thus confirming clonality. The strains differed by their resistance profile which changed remarkably over time (Figure 6 and Table 7). The first MRSA clone identified exhibited resistance to penicillin [*blaZ*], to cefoxitin [*mecA*], to trimethoprim [*dfrK*], to tetracycline [*tet*(M)], to streptomycin [str], to gentamicin and kanamycin [*aac(6')-Ie-aph(2')-Ia*] and to ciprofloxacin [GyrA-Ser83Leu and GrlA-Tyr80Phe]. This clone was detected once in July 2011 and reappeared just two years later, in September 2013; first in a case of a conjunctival infection and was then associated with 21 more infections in the oncoming year, until September 2014 (Figure 6).

All but two of the isolates from the first outbreak showed the same resistance pattern (Table 7). One isolate from July 2014 had additional resistance to chloramphenicol (MIC > 64 mg/L, [cat_{pC221}]) and the last isolate of the first outbreak was susceptible to streptomycin (MIC = 8 mg/L, *str*-negative). No MRSA strains were isolated within the next 8 months. A second MRSA outbreak occurred in May 2015. The first MRSA ST398–t011–SCC$_{mec}$IV isolate from this outbreak (May 2015 to May 2016) had additional resistance to chloramphenicol (MIC > 64 mg/L, [cat_{pC221}]) and the second isolate was also resistant to rifampicin [RpoB His481Asn]. This clone was recovered from 17 other infections in the year following the second outbreak. During that time, only one MRSA ST398–t011–SCC$_{mec}$IV was found to have a different resistance pattern: it was susceptible to chloramphenicol (MIC < 8 mg/L) and streptomycin (MIC = 8 mg/L). Of note, the emergence of MRSA ST398–t011–SCC$_{mec}$IV exhibiting additional resistance to chloramphenicol and rifampicin became frequent after these two antibiotics have been implemented into the MRSA therapy protocols (Figure 6). It should be noted, that the owners of all horses that received chloramphenicol and/or rifampicin declared in the passports of their horses that the animals were excluded from slaughter for human consumption.

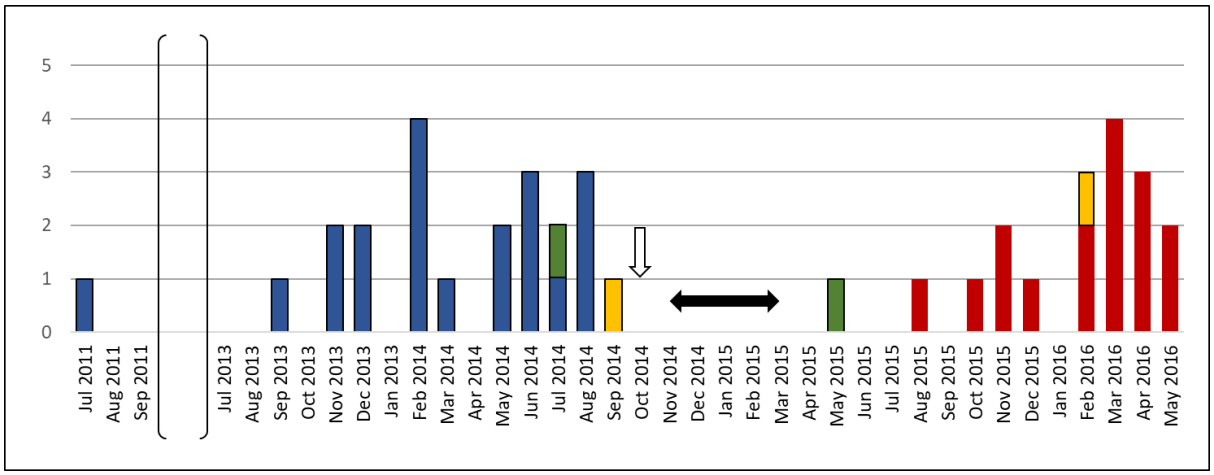

Figure 6 | *Occurrence and frequency of equine MRSA ST398-t011 isolates during the survey period.* Each column represents the number of isolates per month, different column patterns indicate different resistance pattern. : isolates resistant to penicillin, cefoxitin, tetracycline, trimethoprim, streptomycin, gentamicin, kanamycin and ciprofloxacin. : isolates additionally resistant to chloramphenicol. : isolates susceptible to chloramphenicol and streptomycin. : isolates additionally resistant to chloramphenicol and rifampicin. : period without MRSA isolate; : inclusion of chloramphenicol and rifampicin into the antimicrobial treatment protocol.

From the ten MRSA strains isolated from human nasal swabs seven belonged to genotype ST398–t011–SCC$_{mec}$IV and shared the same VNTR pattern demonstrated in MRSA strains of equine origin. Only slight differences could be observed among the strains' resistance profiles (Table 7). Based on genotyping, three human isolates differed both from other human and equine strains. One was isolated from a technician, and it belonged to genotype ST398–t1580–SCC$_{mec}$IV but otherwise showed the same wide-scale resistance pattern as the aforementioned genotype. A third genotype ST541–t1250–SCC$_{mec}$V was isolated from two members of the management who worked occasionally in the stables but not directly with horses. Both isolates shared common VNTR and antimicrobial resistance patterns markedly differing from those observed in case of the ST398 strains (Table 7).

Table 7 | *Genetic characteristics and antibiotic resistance profile of methicillin-resistant* Staphylococcus aureus *isolates from horses and humans*. The MIC breakpoints determining resistance were those recommended for *S. aureus* in CLSI supplement M100-S20, 6 expect for streptomycin for which breakpoint from the French Society for Microbiology (www.sfm.asso.fr) was used. (*) one isolate had both H481S and S529Y mutations in the RpoB protein sequence. MIC, minimum inhibitory concentration; MLVA, multiple-locus variable number tandem repeat analysis; MLST, multilocus sequence type; SCC_{mec}, *Staphylococcus* Cassette Chromosome mec; PEN, penicillin; TET, tetracycline; TMP, trimethoprim; GEN, gentamicin; KAN, kanamycin; CIP, ciprofloxacin; STR, streptomycin; RIF, rifampicin; CHL, chloramphenicol; ERY, erythromycin; CLI, clindamycin.

		Origin		Genotype		Antibiotic resistance phenotype and genotype (resistance breakpoints in mg/L)									
						TET	RIF	STR	GEN-KAN	PEN	CHL	TMP	ERY-CLI	CIP	
						≥16	≥0.5	≥32	≥16/≥64	≥2	≥64	≥32	≥8/≥4	≥8	
Representative Isolate	**Date of Isolation**	**Horse**	**Human**	**MLVA**	**MLST-*spa*-SCC_{mec}**		**RpoB**							**GyrA**	**GrlA**
DL-443-11	Jul 2011	20	0	A	ST398-t011-IV	*tet*(M)	-	*str*	aac6-Ie- aph2-Ia	*blaZ*	-	*dfrK*	-	S83L	S80F
DL-330-14	Jul 2014	1	0	A	ST398-t011-IV	*tet*(M)	-	*str*	aac6-Ie- aph2-Ia	*blaZ*	cat_{pC221}	*dfrK*	-	S83L	S80F
DL-472-14	Sep 2014	1	0	A	ST398-t011-IV	*tet*(M)	-	8	aac6-Ie- aph2-Ia	*blaZ*	-	*dfrK*	-	S83L	S80F
DL-180-15	May 2015	1	0	A	ST398-t011-IV	*tet*(M)	-	*str*	aac6-Ie- aph2-Ia	*blaZ*	cat_{pC221}	*dfrK*	-	S83L	S80F
DL-261-15	Aug 2015	16	1	A	ST398-t011-IV	*tet*(M)	H481S*	*str*	aac6-Ie- aph2-Ia	*blaZ*	cat_{pC221}	*dfrK*	-	S83L	S80F
DL-083-16	Feb 2016	1	3	A	ST398-t011-IV	*tet*(M)	H481S	8	aac6-Ie- aph2-Ia	*blaZ*	-	*dfrK*	-	S83L	S80F
LTK-07-17	Oct 2017	0	1	A	ST398-t011-IV	*tet*(M)	H481S	16	aac6-Ie- aph2-Ia	*blaZ*	cat_{pC221}	*dfrK*	-	S83L	S80F
LTK-05-17	Oct 2017	0	2	A	ST398-t011-IV	*tet*(M)	H481S	*str*	aac6-Ie- aph2-Ia	*blaZ*	-	*dfrK*	-	S83L	S80F
LTK-01-17	Oct 2017	0	1	B	ST398-t1580-IV	*tet*(M)	H481S	*str*	aac6-Ie- aph2-Ia	*blaZ*	cat_{pC221}	*dfrK*	-	S83L	S80F
LTK-12-17	Oct 2017	0	2	C	ST541-t1250-V	*tet*(M)/*tet*(K)	-	16	neg	*blaZ*	*fexA*	-	*erm*C	-	S80Y

5.3.2 Occurrence and characteristics of equine-related MRSA strains in various epidemiological situations

The hygienical screening of 116 horses leaving the DCEM resulted in 54 (46.6%) MRSA isolates, of which 53 was *mecA*-positive, while one isolate harboured the *mecC* gene. Of the 128 incoming horses, eight (6.3%) carried MRSA. Out of those latter eight, seven isolates were identified as MRSA CC398–t011–SCC$_{mec}$IV. All of them were *lukPQ*-positive but lacked both the *lukS-PV/lukF-PV* and *scn* virulence genes. The resistance pattern of these strains was also uniform: besides beta-lactams, the strains were resistant to the investigated aminoglycosides, ciprofloxacin, rifampicin, sulfamethoxazole-trimethoprim, tetracycline, and carried the *str* gene resulting in 0-14 mm zone diameters. The aminoglycoside resistance was conferred by the bifunctional *aac(6')Ie-aph(2")Ia* resistance gene.

One strain was typed as t127, which is attributed to the clonal complex (CC)1, a lineage comprising host generalist MRSA and *S. aureus* strains. This isolate was *lukS-PV/lukF-PV*-negative but carried the *scn*, marker gene of the human-related immune evasion cluster (IEC). This isolate showed a moderate resistance pattern, compared to the CC398 strains, however it was still multiresistant. According to their unique clinical identifier, any of the incoming MRSA-positive horses was neither previously treated nor hospitalized in the DCEM. Further details of the MRSA strains are summarized in Tables 8 and 9.

From the subgroup of 41 horses, which were sampled both upon admission and at their dismissal, 24 (58.5%) horses tested negative both times, 12 (29.3%) were negative upon admission, but left the hospital contaminated with MRSA. Of the five horses, which arrived as MRSA-positive, four tested positive again, while one horse was apparently negative at its dismissal.

Eight of the 12 environmental wipes collected in the DCEM cultured MRSA. We identified at least one MRSA-positive sites per investigated units, including the colic, orthopaedic and isolation stables (n = 5); the two examination halls (one positive sample each), and the central staff room (n = 1). All isolate was typed as CC398–SCC$_{mec}$IVa, *lukS-PV/lukF-PV*- and *scn*-negative, carrying the *lukPQ* gene and the canSNP1748T. The only isolate selected for *spa* typing showed the t011 type.

The country-wide screening of 325 horses of liveries and studs without a recent history of MRSA yielded only MRSA-negative samples. The distribution of farms is shown in Figure 7.

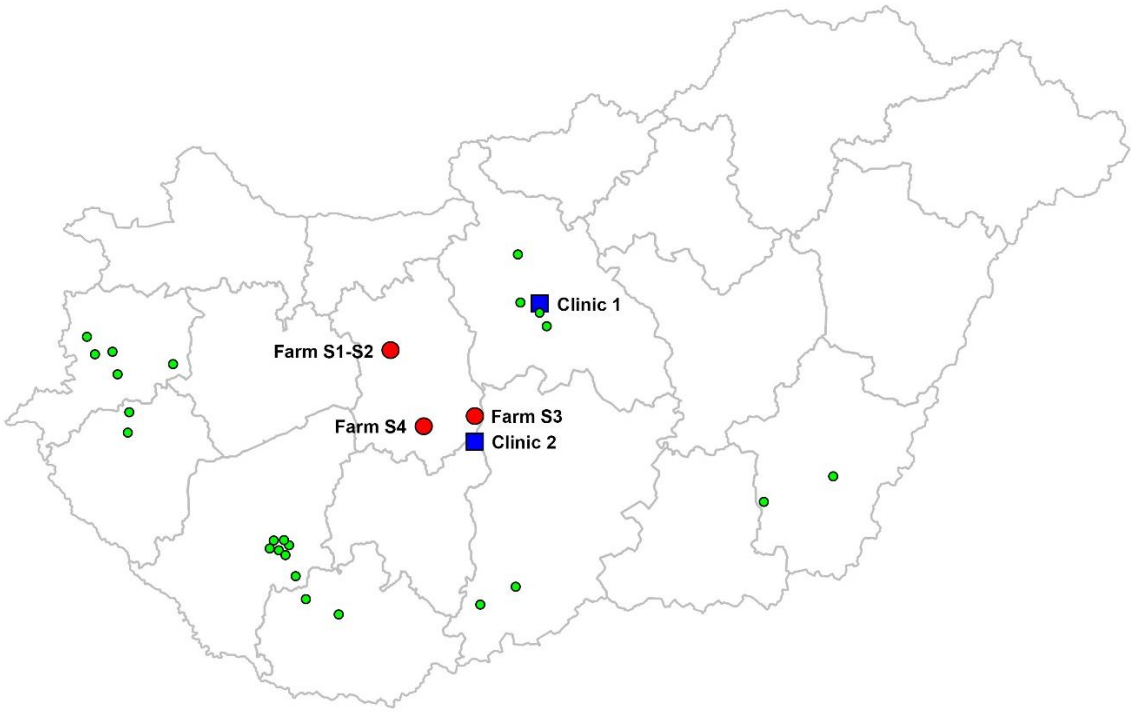

Figure 7 | *Location of clinics and equine farms involved in the study*
Blue square, equine clinic; red circle, farms with recent MRSA outbreak;
green circle, location of equine farms without MRSA in the country-wide surveillance.

The targeted sampling of a veterinary clinic (Clinic 2) with a recent MRSA outbreak resulted in 19 MRSA isolates. All hospitalized horse patients tested positive (n = 5), while 5/19 (26.3%) persons from the staff carried MRSA. Sixty percent (9/15) of the environmental samples were positive, including surgical equipments (3/7) and dust samples collected from the large animal surgical theatre (1/2), the small animal examination room (3/3), the laboratory (1/1) and the changing room (1/2). All isolates showed the genetic characteristics of EHA-MRSA CC398, carried the *lukPQ* gene but lacked both the *scn* and *lukS-PV/lukF-PV* genes. The selected strains were identified as *spa* type t011. The resistance pattern differed slightly among the isolates, however, it divided them into two distinct resistance groups. While all isolates were resistant to beta-lactams, ciprofloxacin, all tested aminoglycosides, sulfamethoxazole-trimethoprim, tetracycline and carried the *str* gene, only 13 showed additional resistance to rifampicin and the remaining six isolates were, additionally, resistant to chloramphenicol only, conferred by the cat_{pC221} gene (Table 9).

The MRSA strain isolated from the index case of this outbreak (E02871800) was typed as *lukPQ*-positive EHA-MRSA CC398, with the same resistance pattern identified in Clinic 2, harbouring only the additional rifampicin resistance. At Farm S1 out of 71 horses no further one was tested MRSA-positive. From the ten persons at Farm S1 two carried the same EHA-MRSA CC398, and this genotype was also identified in the case of the three isolates recovered

from the environmental samples (3/6); all of them were *lukPQ*-positive and showed the same resistance pattern as described above. At Farm S2, four out of 49 (8.2%) sampled horses carried the EHA-MRSA CC398 and this type was identified in the single human (1/4) and environmental (1/27) sample, respectively. The strains' resistance pattern was also very similar to the aforementioned ones and only single isolates showed an additional resistance to fusidic acid or linezolid. Table 9 shows the further characteristics of the isolates. At Farm S3, we could only sample the horses, of which none carried MRSA (0/26).

A total of 322 samples from Farm S4, with an apparently isolated outbreak yielded 30 (9,3%) MRSA strains. Nine from 178 horses were tested positive at least once (5.0%). Of these, two horses were positive again at their consecutive control sampling, resulting in a total of 11 equine strains. All isolates were EHA-MRSA CC398, of which one strain lacked all investigated virulence genes, the others carried the *lukPQ* gene. The sampling of 26 distinct environmental sites yielded five (23.1%) MRSA isolates on the primary testing. From the environmental control samples (n = 13) only a box of a positive horse was proven to be still contaminated with MRSA. All environment originated isolates were characterised as EHA-MRSA CC398 and *lukPQ*-positive. From the 59 tested staff and household members 12 (20.3%) were positive. Of note, every positive person tested positive only once, since they underwent a decolonisation therapy after the first positive sample. The genotypes of human isolates showed a marked variability: eight isolates belonged to the EHA-MRSA CC398 lineage, whereas three were classified as SCC_{mec}V and were t011 (n = 2) or t034 (n = 1) by *spa* typing. A single isolate was t2298 and SCC_{mec}IVa and belonged to CC30. Among the EHA-MRSA CC398 isolates, two major resistance patterns could be distinguished: eight isolates were resistant to beta-lactams, ciprofloxacin, all tested aminoglycosides, sulfamethoxazole-trimethoprim, tetracycline and carried the *str* gene, and 16 isolates showed additional resistance to erythromycin and clindamycin, conferred by the *erm*(C) gene. Other resistances occurred sporadically among the equine hospital-associated isolates. The isolates with distinct genotypes differed markedly from the EHA strains by their resistance patterns as well. The distribution of isolates in different genotypes and resistance patterns are detailed in Table 9.

Table 8 | *Proportion of methicillin-resistant* Staphylococcus aureus *positive sampled horses, individuals and sites and basic genetic characteristics of the isolated strains in various epidemiological situations.* ST, sequence type; CC, clonal complex of multilocus sequence types; *spa*, *Staphylococcus aureus* protein A sequence type, SCC_{mec}, *Staphylococcus* cassette chromosome mec type. (*) One representative strain was selected for *spa* typing

Place	Time	Origin	MRSA-positive/ Number of Individuals or Sites	Genotype ST/CC-*spa*-SCCmec (Number of Isolates)
Hygienical Screening of The DCEM				
Clinic 1 (DCEM)	Nov 2017 - Dec 2018	leaving horses	54/116 (46.6%)	*mecA*-positive MRSA (n = 53) *mecC*-positive MRSA (n =1)
	May - Dec 2018	incoming horses	8/128 (6.3%)	CC398-t011-IVa (n = 7) ST1-t127-IVa (n = 1)
	Jan 2018	environment	8/12 (66.7%)	CC398-t011-IVa*
Country-Wide Prevalence Survey				
	Apr 2018 - Feb 2019	horse	0/325 (0.0%)	-
Targeted Sampling of an Animal Clinic with a Recent Nosocomial Outbreak and Related Equine Farms				
Clinic 2	May - Jun 2018	horse	5/5 (100%)	CC398-t011-IVa
		human	5/19 (26.3%)	CC398-t011-IVa
		environment	9/15 (60%)	CC398-t011-IVa*
Farm S1	May 2018	horse	0/71 (0.0%)	CC398-t011-IVa
		human	2/10 (20.0%)	CC398-t011-IVa
		environment	3/6 (50.0%)	CC398-t011-IVa*
Farm S2	Jun 2018	horse	4/49 (8.2%)	CC398-t011-IVa
		human	1/4 (25.0%)	CC398-t011-IVa
		environment	1/27 (3.7%)	CC398-t011-IVa
Farm S3		horse	0/26 (0.0%)	-
Targeted Salmpling of a Stud with an Apparently Unrelated Outbreak				
Farm S4	Feb 2019 - Apr 2019	horse	9/173 (5.2%)	CC398-t011-IVa
		human	12/54 (22.2%)	CC398-t011-IVa (n = 8) CC398-t011-Vc (n = 2) CC398-t034-Vc (n = 1) non-CC398, t2298, *scn*-positive (n = 1)
		environment	6/26 (23.1%)	CC398-t011-IVa*

.

Table 9 | *Resistance patterns and major virulence traits of methicillin-resistant Staphylococcus aureus strains isolated in various epidemiological situations.* CHL, chloramphenicol; CIP, ciprofloxacin; CN, gentamicin; DA, clindamycin; E, erythromycin; FA, fusidic acid; K, kanamycin; LNZ, linezolid; P, penicillin; SYN, quinupristin-dalfopristin; RA, rifampicin; S, streptomycin; SXT, trimethoprim and sulfamethoxazole; TE, tetracycline; TIA, tiamulin, TGC, tigecycline; TOB, tobramycin;; CC, clonal complex of multilocus sequence types; *spa*, *Staphylococcus aureus* protein A sequence type, SCC_{mec}, *Staphylococcus* cassette chromosome mec type; *str*, streptomycin resistance gene; $catp_{C221}$, chloramphenicol acetyltransferase gene encoded on plasmid *pC221*; *erm(C)*, rRNA adenine N-6-methyltransferase gene; *fexA*, chloramphenicol/florfenicol exporter gene; *lukPQ*, bicomponent leukocidin LukPQ gene; scn, human-associated *Staphylococcus* complement inhibitor (SCIN) gene. (*)The number of strains showing resistance or intermediate resistance indicated in uppercase brackets. (**) The phenotypic result was confirmed by testing all isolates for the bifunctional aminoglycoside acetyltransferase and phospotransferase (AAC/APH) gene [*aac(6')Ie-aph(2")Ia*]. No discrepancies were observed. (***) The number of strains harbouring the virulence gene is indicated in brackets.

Number of Isolates (Horses/ Humans/Environment)	Genotype (CC-*spa* type-SCCmec type)	CHL	CIP	DA	E	FA*	CN**	K	P	SYN*	RA	S	SXT	TE	TIAMU	TOB	Resistance Genes	Vrulence Genes***
Isolates of the Hygienical Screening of the DCEM																		
7 (7/0/0)	CC398-t011-SCC_{mec}IVa		■				■	■	■		■	*str*	■	■		■		*lukPQ (6)*
1 (1/0/0)	CC1-t127-SCC_{mec}IVa			■	■			■	■			*str*		■				*scn*
Targeted Sampling of a Clinic and Related Farms with a Recent Nosocomial Outbreak (Clinic 2 and Farms S1 and S2)																		
13 (3/2/8)	CC398-t011-SCC_{mec}IVa		■				■	■	■		■	*str*	■	■		■		*lukPQ*
6 (2/3/1)	CC398-t011-SCC_{mec}IVa	■	■				■	■	■			*str*	■	■		■	cat_{pC221}	*lukPQ*
Farm S1																		
1 (1/0/0)	CC398-t011-SCC_{mec}IVa		■				■	■	■		■	*str*	■	■		■		*lukPQ*
5 (0/2/3)	CC398-t011-SCC_{mec}IVa		■				■	■	■		■	*str*	■	■		■		*lukPQ*
Farm S2																		
6 (4/1/1)	CC398-t011-SCC_{mec}IVa		■			■ (1)	■	■	■	▣ (1)	■	*str*	■	■		■		*lukPQ*
Targeted Salmpling of a Stud with an Apparently Unrelated Outbreak (Farm S4)																		
8 (4/3/1)	CC398-t011-SCC_{mec}IVa		■				■	■	■		■	*str*	■	■		■		*lukPQ (7)*
16 (6/5/5)	CC398-t011-SCC_{mec}IVa		■	■	■	■ (3)	■	■	■	▣ (1)	■	*str*	■	■		■	*erm(C)*	*lukPQ*
1 (0/1/0)	CC398-t011-SCC_{mec}V		■		▣		■	■	■		■					■		-
1 (0/1/0)	CC398-t011-SCC_{mec}V	■	■	■					■		▣	*str*		■			*fexA*	-
1 (0/1/0)	CC398-t034-SCC_{mec}V			■				■	■	▣		*str*	■	■	■			-
1 (0/1/0)	CC30-t2298-SCC_{mec}IVa				▣			■	■					■		■		*scn*

5.3.3 Whole genome sequence-based molecular epidemiologic investigations of equine-related MRSA CC398 strains isolated between 2008-2019 in Hungary

The second retrospective investigation of Staphylococcus sp. strains originating from the Department and Clinic of Equine Medicine

The systematic, documented conservation of bacterial isolates started in 2009 in the Diagnostic Laboratory, though only seven *Staphylococcus* sp. strains were conserved in this particular year. The isolates originated from five horse patients. From this year there was no comprehensive diagnostic summary available for further evaluation. However, in the timeframe between 2010 and 2018 the diagnostic records consist of 757 discharge or nasal swab samples originating from 744 horses. Of these 744 patients, 189 (25.4%) yielded *Staphylococcus* sp. isolate, at least upon one sampling occasion. From the *Staphylococcus* positive cases, 166 *Staphylococcus* sp. strains were conserved. Together with the seven isolates from 2009 the total of 173 conserved *Staphylococcus* sp. isolates originated from 147 horses.

We investigated the conserved isolates to confirm the primary microbiological results and to characterise the strains. By the applied mPCR1 112 (64.7%) isolates confirmed as *S. aureus*, 61 (41.5%) were *spa*-negative and considered as other *Staphylococcus* sp. Of the 112 *S. aureus* 102 (91.1%) carried the *mecA* gene and all but one was typed as SCC$_{mec}$IVa and MRSA CC398 by the second PCR test, including the 40 strains previously investigated equine MRSA strains. Of note, the 102 MRSA-positive samples originated from 85 horses. Besides the already characterised 40 MRSA strains of the first outbreak investigations, a further 13 isolates were *spa*-typed and showed exclusively type t011. The remaining 48 MRSA CC398-SCC$_{mec}$IVa was confirmed as equine hospital-associated by the SNP1748T MAMA PCR test. The only isolate being both CC398 and SCC$_{mec}$IVa-negative was excluded from further investigations.

In 2017 only three MRSA isolates were conserved. Another eight *S. aureus* strains were confirmed as methicillin resistant based on selective culturing and tested *mecA* positive by subsequent PCR, making the total number of MRSA isolates to 11 in that particular year. The eight isolates were later lost due to technical issues, thus could not be investigated further. Altogether 92 horses were confirmed as MRSA positive between 2009–2018.

By observing the distribution of MRSA positive cases in the timescale, relative moderate annual case numbers (n = 3–6) and MRSA/sampled patient proportions (3.6–7.3%) can be seen between January 2010 and November 2013, mostly separated by longer case-free periods. This tendency was only disrupted by a marked accumulation of cases from July till September 2011 (n = 5). From November 2013 a period characterised by the continuous residence of the pathogen with high MRSA/sampled patient proportions (11.9–24%) followed.

There were only three longer case-free periods thereafter, one in 2014, lasted for 3 consecutive months and two in 2017, each lasted for 2 months. The annual distribution of sampled patients, *Staphylococcus* sp., *S. aureus* and MRSA positive cases and their respective proportions are summarized in Table 10. A monthly distribution of *Staphylococcus* sp. positive cases can be found among the Supplementary Materials. (Supplementary Figure S3)

Table 10 | *Annual distribution of equine-related microbiological data.* (*) In 2016 and 2017 horses were frequently sampled on screening purposes above the screening project, markedly increasing the number of sampled patients. However no further information was provided on the submission forms which would have allowed the differentiation of such cases from those of clinical relevance.

	2010	2011	2012	2013	2014	2015	2016	2017	2018
number of sampled patients with discharge	58	59	83	82	100	46	160*	77*	79
number of *Staphylococcus* sp.-positive cases	8	12	7	12	25	19	60	20	26
S. aureus-positive cases (incl. MRSA-cases)	4	8	3	6	15	11	21	11	19
MRSA-positive cases confirmed	3	5	3	6	14	11	19	11	19
MRSA-positive case/sampled patients with discharge (%)	5.2	8.5	3.6	7.3	14.0	23.9	11.9*	14.3*	24.1

Regarding the clinical outcomes of the cases, we considered the most severe outcome which was recorded on the submission forms. The distribution of sampling sites yielding MRSA positive samples are outlined in Figure 8.

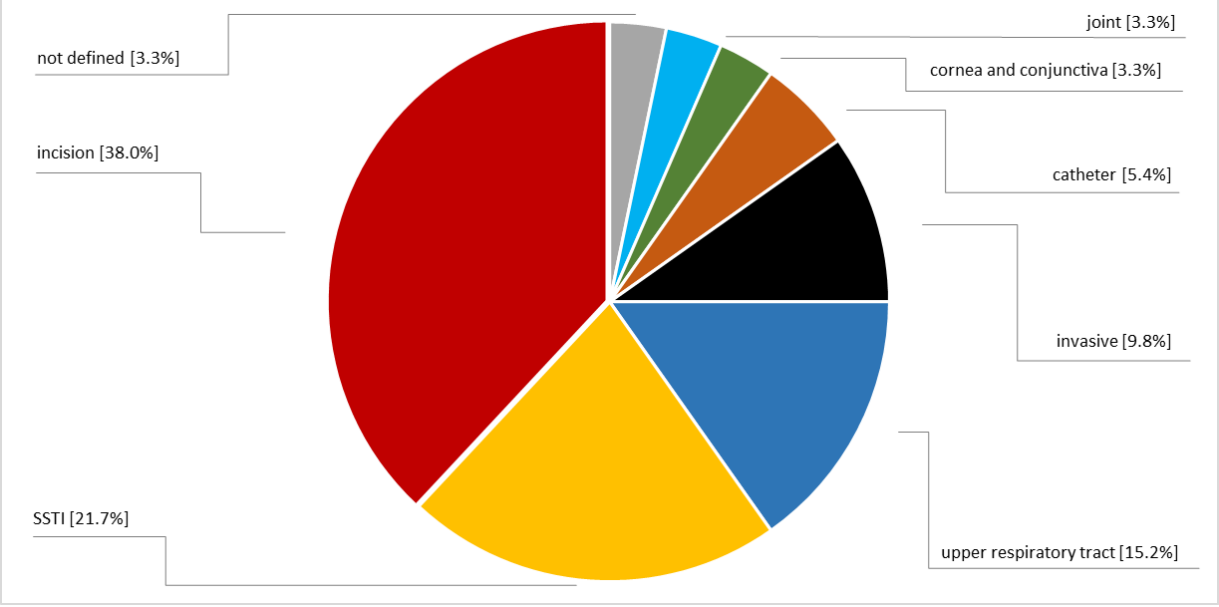

Figure 8 | *Distribution of sampling sites yielding methicillin-resistant* Staphylococcus aureus *in horses hospitalized in the Department and Clinic of Equine Medicine between 2010 and 2018.*
One sampling site per patient was considered. If more sampling sites yielded MRSA, the one with the most severe clinical relevance was involved.

According to the inclusion criteria outlined in the Materials and Methods section, a final set of 15 DCEM isolates were selected for WGS, of which seven originated from the first outbreak investigations and eight were identified during the second attempt (Supplementary Table S3).

Altogether 50 equine-related MRSA strains were subjected to whole genome sequencing. All isolates fulfilled the minimum quality criteria required by the BioNumerics software for the whole-genome MLST analysis. The average denovo coverage of the genomes varied between 47–373, while the average locus coverage between 20–218, providing a satisfactory level of reliability. For further details of the quality criteria please see Supplementary Table S1.

Whole genome-based typing, virulence genes and resistance determinants

The initial analysis of genomes confirmed the results obtained by classical methods: all isolates belonged to the multilocus sequence type 398, *spa* type t011 and SCC_{mec} type IV and none of them carried the Panton-Valentine leukocidin gene (*lukS-PV/lukF-PV*), nor genes of the human-associated immune-evasion cluster (IEC). This latter finding was reinforced by the presence of an intact beta-hemolysin gene (*hlb*) too, which usually serves as an integration site for the IEC-carrying phages. The in-silico analysis also confirmed the presence of the equine lineage-specific 1748T SNP allele variant in all isolates.

All isolates carried the *S. aureus* pathogenicity island bov5 (SaPIbov5), but all lacked the SaPIbov4, and the biocomponent leukocidin genes, *lukMF'* and *lukED*. Thirty-two isolates (64%) harboured the *lukPQ* gene, along with the equid-associated scn_{eq}, showing a 99.9% and 98.3% pairwise identity with the corresponding genes of the reference sequences LT671578, respectively. They were encoded in close proximity to each other on an incomplete prophage of 35 Kb size. The prophage markedly differed from the reference sequence (LT671578; pairwise identity: 69.0%) but showed a high degree of pairwise identity (93.4%) within the Hungarian isolates. In this case, the differences were accumulated in a 4.6 Kb long part (pairwise identity: 42.1%), on the opposite end where the leukocidin and scn_{eq} were encoded. Another phage-associated virulence determinant was discovered in eight isolates. The recently discovered cell-wall teichoic acid modifying enzyme gene *tarP*, which was encoded on a 64.1 Kb size prophage and the gene showed 98.9% pairwise identity with the reference sequence (NC_004740).

The isolates showed a high similarity regarding their investigated resistance gene content and genotypic resistance was in good agreement with the phenotypic profiles in all cases. Besides beta-lactams [*mecA* and *blaZ*], all strain was resistant to tetracycline [*tet*(M)], trimethoprim [*dfrK*], aminoglycosides [*aac(6')Ie-aph(2")Ia*], and – except two isolates (E04721400, E04351600) – carried the plasmid-borne *str* gene, conferring resistance to streptomycin. The decreased phenotypic susceptibility towards fluoroquinolones was also observed in most

strains (46/50). It was conferred either by the already described two amino acid substitutions (n = 42; [GrlA S80F and GyrA S84L]) and resulted in marked phenotypic resistance (d = 0–11 mm) or by another SNP in the *grlA* gene (n = 4; [S80Y]), which manifested as moderate phenotypic resistance (d = 13–20 mm) towards ciprofloxacin. The mutations in the chromosomal *rpoB* gene were identified again: [H481N] in 24 and [S486L] in one case, resulting in decreased susceptibility towards rifampicin. In this selection only eight isolates carried the chloramphenicol resistance gene cat_{pC221} and all of them were resistant to chloramphenicol. Five isolates also harboured the multiresistance gene *erm*(C) and were resistant to erythromycin and clindamycin. A resistance gene [*qacJ*], which is thought to confer resistance quaternary ammonium compounds, occurred in 16 of 50 isolates. All latter three resistance genes were encoded on different plasmids.

By investigating the genetic relatedness, we identified a minimum of 97% of the standardised 1861 core genome loci (maximum: 99%; average: 98.4%) in all isolates. A larger subset of 2424 loci, expectedly increasing the resolution of the analysis, was also defined, representing the maximum common genome (mcg) of the 50 strains. However, both locus subsets resulted in highly similar UPGMA tree topologies, as shown in Figures 9 and 10. There was no observable re-clustering of isolates after the involvement of more MLST loci, hence only the core-genome MLST tree is discussed in details further on. Based on the suggested 24-allele difference cut-off value (Schürch et al., 2018), two distantly related genetic lineages (L1 [n = 8] and L2 [n = 42]) could be distinguished, separated from each other by a range of 37.6–59.6 allelic differences. The within lineage differences of isolates varied between 1.0–10.1 in the case of L1 and 0.0–22.2 in the case of L2. However, L2 seemed to further split into two subclades L2a (n = 9) and L2b (n = 33), which groups showed an average of 16.4 (7.1–22.2) allele difference. Again, the within sub-lineage differences were lower: 0.0–11.1 in L2a and 0.0–14.1 in L2b.

The isolates seemingly split into different genetic clusters according to their time of isolation. L1 consisted of isolates exclusively from the period of 2008–2011. The isolates of L2a were all from the timeframe of 2011 - 2013 while L2b consisted of isolates from 2013 and onwards. The place of origin did not play role in the clustering. The strains from separate locations were randomly clustered into the different lineages within the same time-period and the isolates from both investigated clinics were represented in all genetically distinct groups (Figure 9).

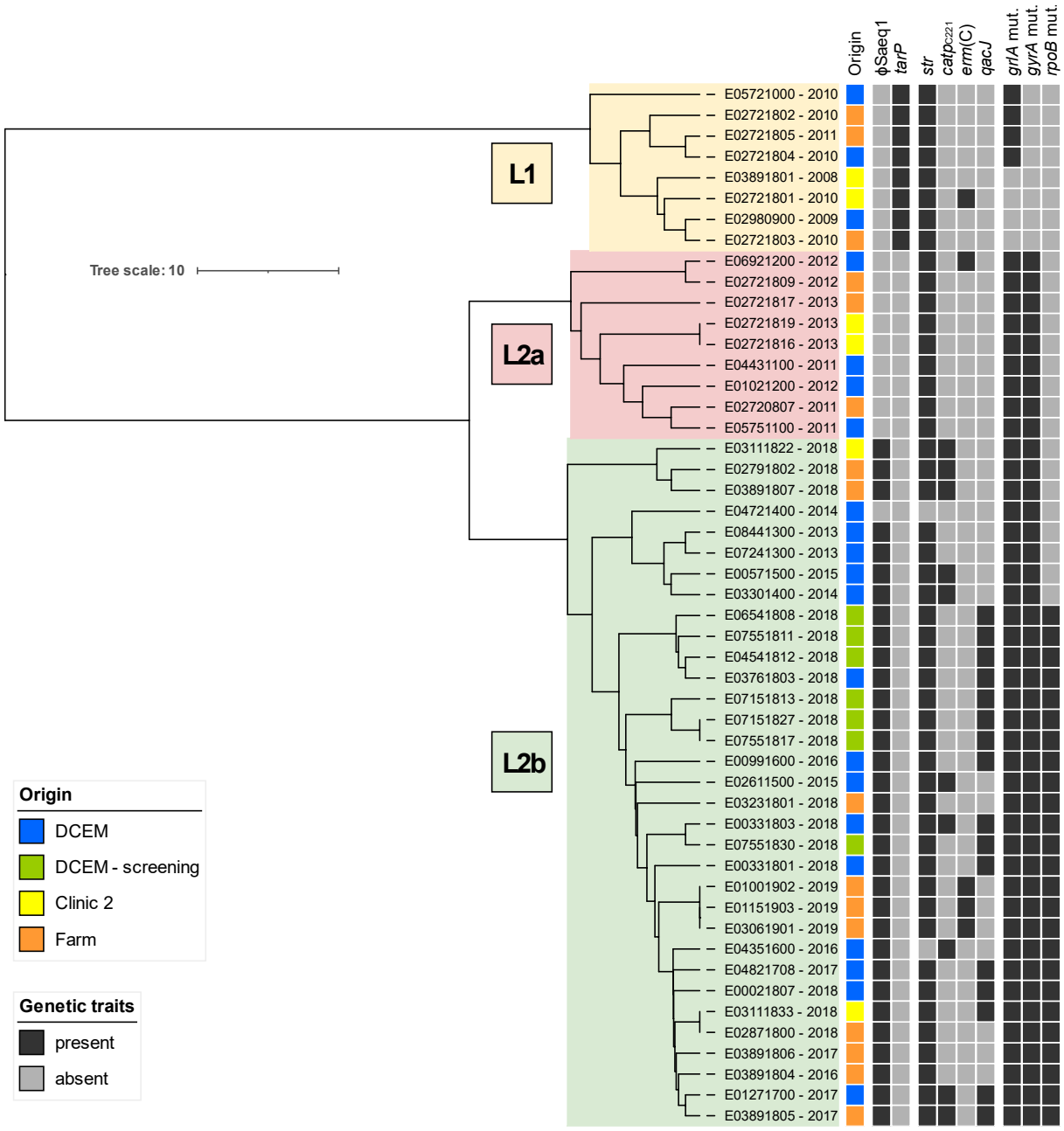

Figure 9 | *Genetic properties and relatedness of MRSA strains isolated from horses and their environment in Hungary.* The unrooted UPGMA tree was generated using the cgMLST distance matrix equine-associated MRSA CC398 isolates. The colour coded annotations show some key feature of the strains. Genetic traits: a wall teichoic acid modifying enzyme, TarP gene (*tarP*), bacteriophage Saeq1 (ϕSaeq1), streptomycin resistance gene *str*, chloramphenicol acetyltransferase gene $catp_{C221}$, rRNA adenine N-6-methyltransferase gene *erm*(C), resistance gene to quaternary ammonium compounds *qacJ*, chromosomal point mutations conferring resistance to fluoroquinolones (*grlA* and *gyrA* mut.) or to rifampicin (*rpoB* mut.). The identified genetic clusters are also highlighted: ▭, lineage 1 (L1); ▭, lineage 2a, and ▭ lineage 2b (L2a and L2b). Scale bar indicates 10 allelic differences. Please note, that clusters L1, L2a and L2b are arranged according to the time of isolation and not according to the place of origin, while strains within the different subclusters are highly clonal (<15 core genome allele difference).

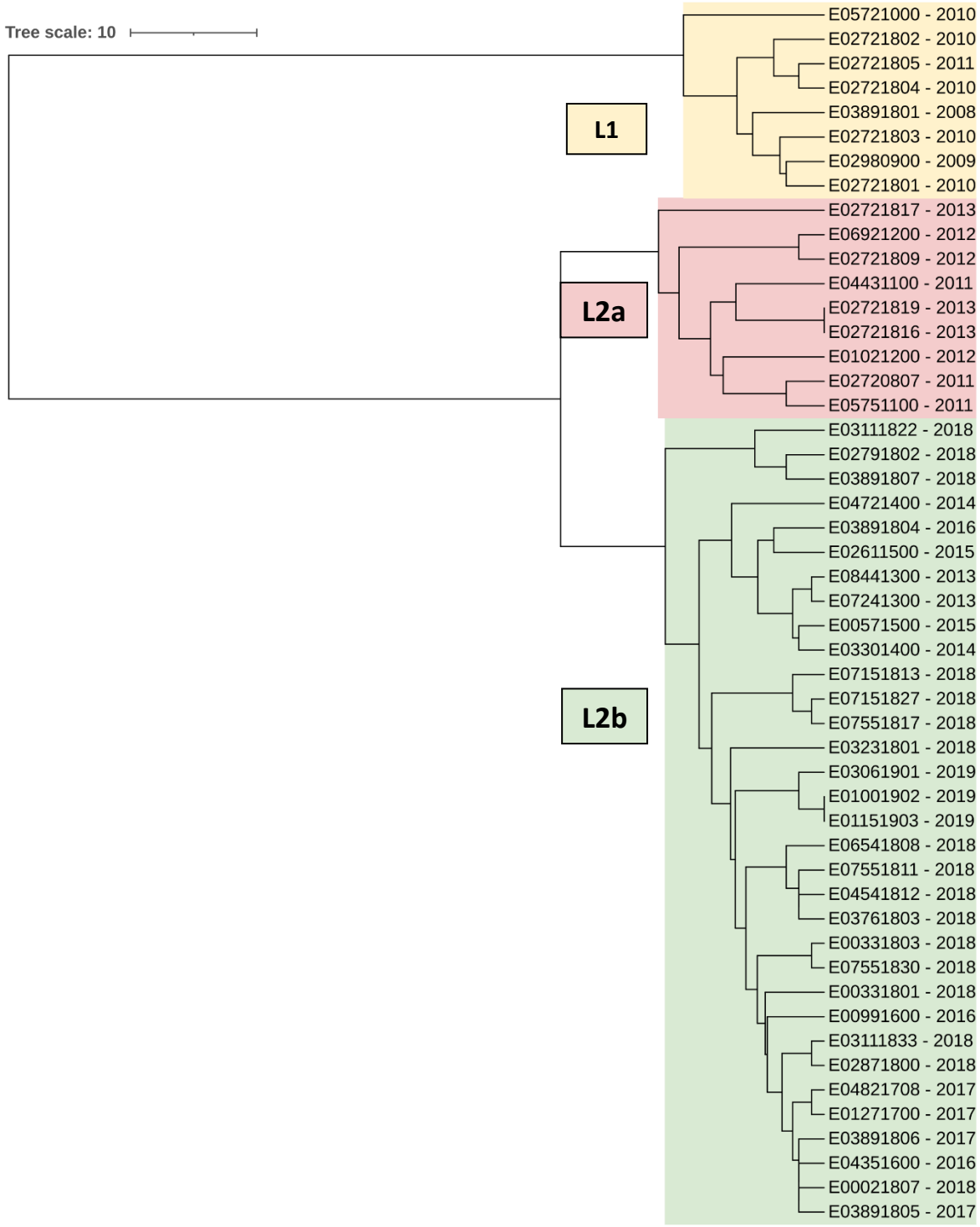

Figure 10 | *Genetic relatedness of MRSA strains isolated from horses and their environment in Hungary.* The unrooted UPGMA tree was generated using the maximum common genome (mcg)MLST distance matrix of equine-associated *MRSA CC398* isolates. The annotation shows the identified genetic clusters: ▭, lineage 1 (L1); ▭, lineage 2a, and ▭ lineage 2b (L2a and L2b). Scale bar indicates 10 allele difference. Please note, that the tree is in good agreement with that in Figure 9. No re-clustering of strains can be observed.

Interestingly, the occurrence of some virulence and resistance determinants followed the genetic clustering of isolates. Only L1 isolates harboured the *tarP* gene and half of them (n = 4) also had the single *grlA* S80Y mutation, resulting in moderate ciprofloxacin resistance. Of note, these resistant isolates were all either isolated in the DCEM or were recovered from animals recently treated in the clinic in years 2010 and 2011. In contrast to L1, the isolates of L2 missed the *tarP* gene but had SNPs on both genes *grlA* and *gyrA*. The separation of L2b (n = 33) from L2a (n = 9) was further supported by the emergence of the horse-specific leukocidin gene *lukPQ*, which is occurred in all but one isolate of L2b. Three resistance determinants also occurred only in the strains of L2b: cat_{pC221} (9/33) and *qacJ* (16/33), and the mutations in the chromosomal *rpoB* gene (H481N, 24/33; S486L, 1/33).

In accordance with the WGS results, the emergence of some abovementioned genetic and phenotypic traits also showed a time-related change in the clonal lineages' gene content by the investigation of all conserved DCEM clinical isolates. We tested a total of 102 isolates for the presence of *lukPQ*, cat_{pC221}, and *qacJ*. We also investigated the phenotypic rifampicin resistance of strains either retrospectively or by re-culturing and subsequent testing. Until 2013 none of the traits have occurred. In the timeframe of interest (January 2013 – December 2018), 86 isolates were investigated, which were derived from 72 horses. *lukPQ* was the first trait which emerged in 2013 and became prevalent thereafter, occurring in 90.7% of the isolates (78/86). Of note, the eight negative isolates were cultured from seven horses' sample, of which one animal later yielded several *lukPQ*-positive MRSA isolates. The chloramphenicol resistance gene cat_{pC221} was first detected in an isolate from July 2014 but spread only after 2015: its prevalence reached as high as 85.7% (36/42) between January 2015 and December 2017, then it decreased to 28.6% (6/21) in the following year. The *qacJ* gene emerged in February 2015 and became prevalent rapidly with 80.1% (51/63) of the isolates carrying it. The emergence of phenotypic rifampicin resistance had a similar pattern to that of *lukPQ* and *qacJ*. The first resistant isolate was cultured in August 2015, and all of them showed decreased susceptibility onwards, including the eight lost isolates from 2017 (96.4%; 54/56). Of note, there were two isolates from August 2016, originating from two distinct horses, which carried none of the concerned mobile genetic elements and one of them were also rifampicin-sensitive. The distribution of the certain traits among the isolates is outlined in Figure 11.

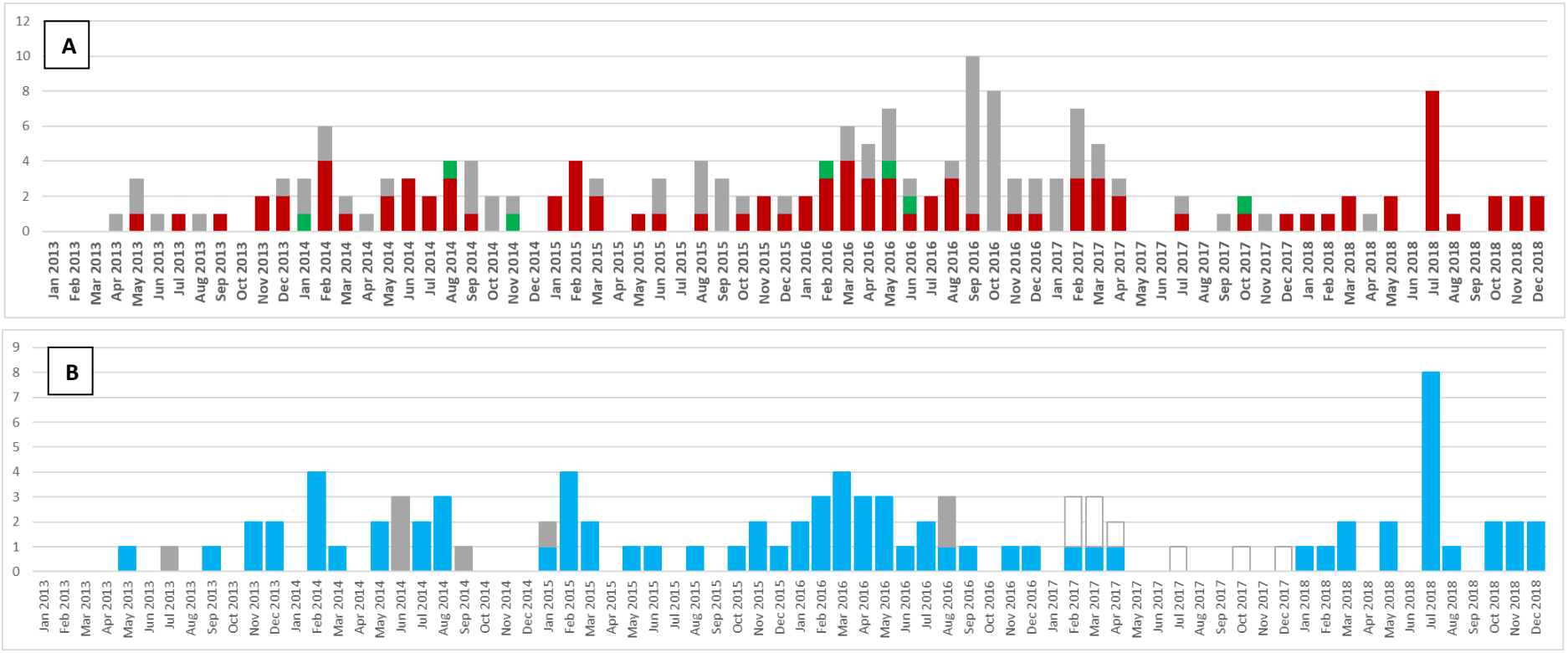

Figure 11 | *Distribution of equine Staphylococcus isolates per month between January 2013 and December 2018 and the emergence of selected genetic and phenotypic traits in the Department and Clinic of Equine Medicine*. A) Occurrence of *Staphylococcus* sp. ( ), methicillin-sensitive ( ) and methicillin resistant ( ) *S. aureus* strains. B) Presence ( ) or absence ( ) of the equine-specific leukocidin gene, *lukPQ*. Columns left blank indicate missing typing data.

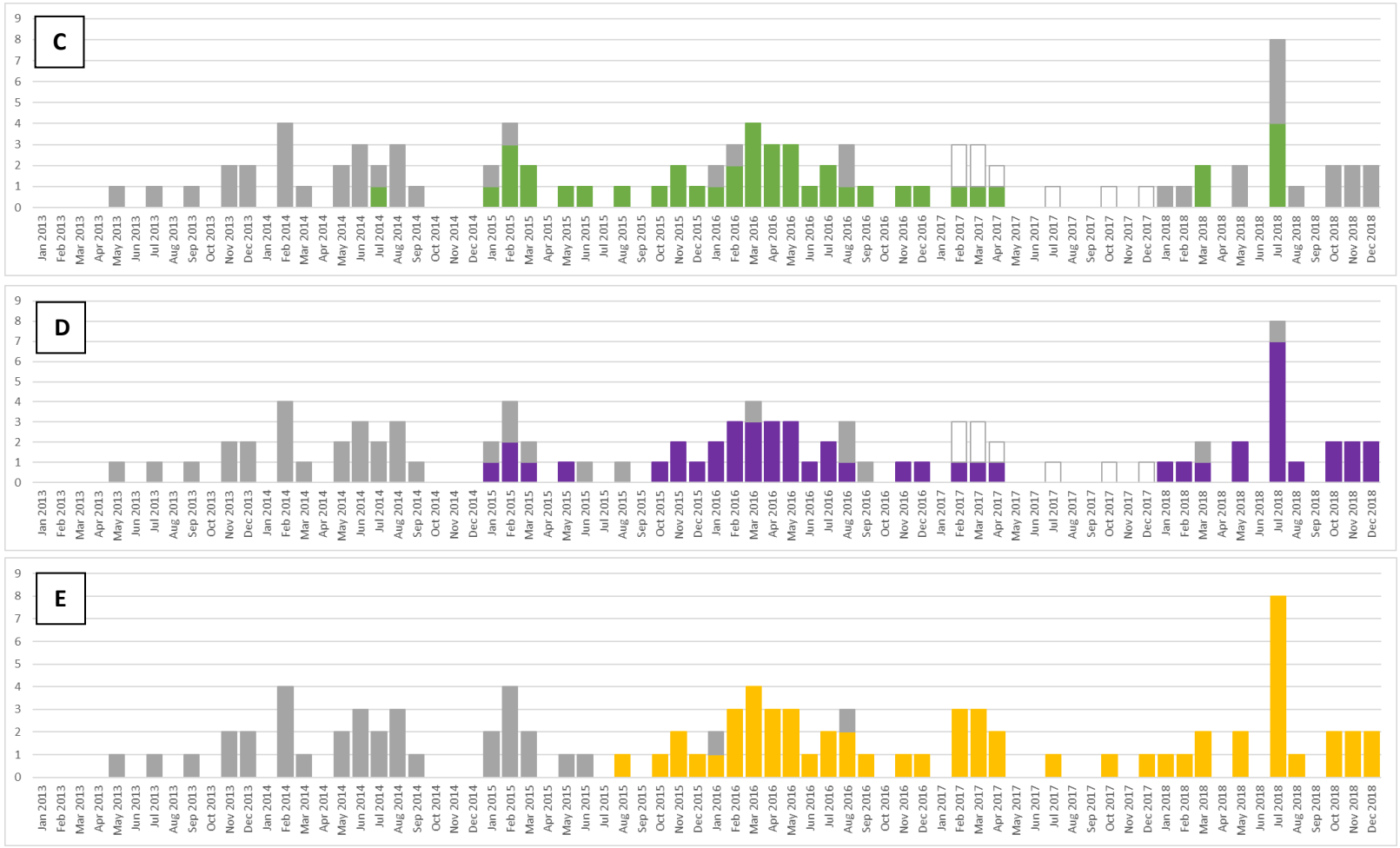

Figure 11 (continued) | *Distribution of equine Staphylococcus isolates per month between January 2013 and December 2018 and the emergence of selected genetic and phenotypic traits in the Department and Clinic of Equine Medicine.* C) Presence ( ) or absence ( ) of the chloramphenicol acetyltransferase gene cat_{pC221}; D) Presence ( ) or absence ( ) of the quaternary ammonium compound resistance gene *qacJ*; D) Presence ( ) or absence ( ) of the rifampicin resistance based on either phenotypic investigations or supplemented with genetic data. Columns left blank indicate missing typing data.

To evaluate the degree of genetic relatedness of our equine hospital-related strain collection, we have selected whole genome sequence data of EHA-MRSA isolates originating from different parts of Europe and compared them to the Hungarian dataset. The WGS data of 99 European isolates were appropriate for evaluation, making the total number of analysed EHA-MRSA strains 149. The majority of strains was from three countries, Austria (n = 46), Germany (n = 25) and Denmark (n = 13), all isolated in the timeframe of 2013-2018. A few other datasets originated from Switzerland (n = 8; 2017), The United Kingdom (n = 3; 2008), *Spa*in (n = 2; 2015 and 2011), Belgium, and The Netherlands (one each; 2008 and 2010).

The pairwise allelic differences between isolates ranged from zero up to 82.5 (median: 39.5) and the resulting UPGMA tree showed a rather heterogenous structure, composed of more groups of closely related isolate clusters (allelic difference < 24). However, the first branching apparently separated the strains into two main clades. One comprised almost all North-Western European, a few Austrian and German isolates and all Hungarian L1 strains. Despite of their various origin, most strains in this clade carried the *tarP* gene (88.4%; 38/43), similarly to the L1 isolates. The other clade was made up of isolates from Austria, Germany, a few Swiss isolates and the Hungarian L2 strains and assigned as Middle-European clade. This group consisted of more isolates from a single country, and most of them formed a few closely related clusters, characterised by similar properties.

By the involvement of the European isolates, strains of the Hungarian L1 and L2 separated more markedly from each other and clustered into the two different clades of the UPGMA tree, as it was already demonstrated. The separation of sub-clusters L2a and L2b became also more pronounced. The strains of L2a and a group of Austrian isolates (n = 7) from 2013–2017 formed a new sub-clade by sharing 12.1–20.01 (median: 17.2) allele differences, considered as a similar distance that separated L2a from L2b (7.1–22.2, median: 17.2).

Some genetic traits, that frequently occurred in the Hungarian isolates, were rare amongst the other European isolates and vice versa. We could demonstrate *lukPQ* in only four German and in an Austrian isolate (IDs: IMT37082, IMT37325, IMT37340, IMT37510 and 3214/2017). All *lukPQ*-carrying German isolates belonged to one, separate cluster in the North-Western clade of the UPGMA tree, and the Austrian isolate also appeared more divergent compared to the Hungarian *lukPQ*-positive isolates. The resistance gene *qacJ* was present in a singleton from Austria (ID: 2646/2017), and the cat_{pC221} could not be detected in any other isolates, except the Hungarian strains. On the other hand, the genes of IEC occurred in some highly clonal, but otherwise distantly related group of isolates from Denmark and Germany, and in a singleton from Switzerland. As it was already suggested, *tarP* is rather the feature of the ‘North-Western’ group, however it occurred in an Austrian clonal lineage of 13 isolates and in further two strains

closely related to the Hungarian L2a group. The occurrence of *erm*(C) was rare both among the Hungarian and other European isolates. Only three Austrian isolates (2202/2015, 3855/2014, 3214/2017), distantly related to the Hungarian lineages, carried the resistance gene and its plasmid. The UPGMA tree depicting the relatedness of the Hungarian and other European isolates annotated with selected genetic features is outlined in Figure 12.

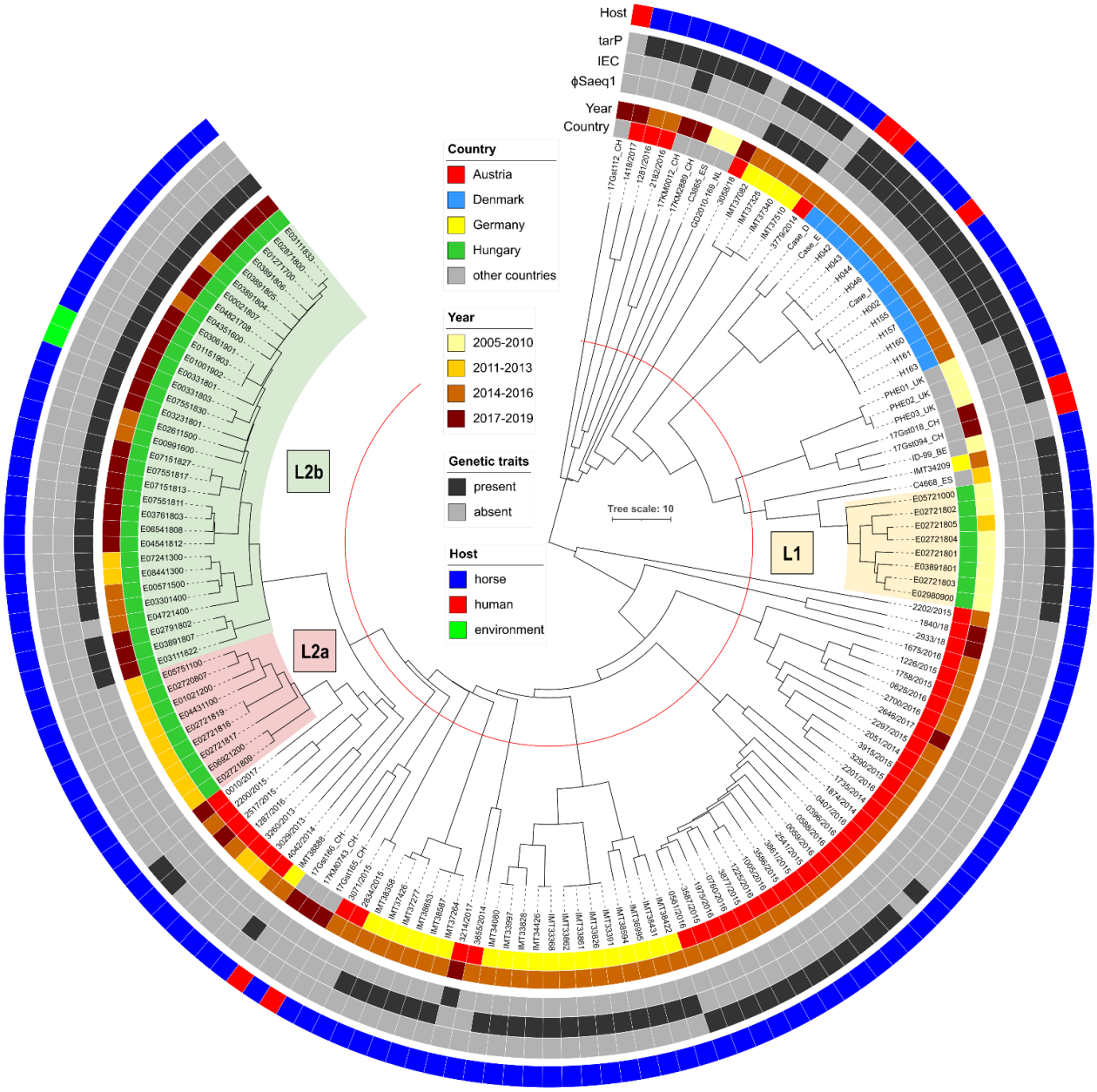

Figure 12 | *The Hungarian equine-related methicillin-resistant Staphylococcus aureus strains in the context of other European isolates.* The UPGMA tree was generated using the cgMLST distance matrix of equine-associated *MRSA CC398* isolates. The annotations show some key feature of the strains by colour codes. The identified Hungarian equine MRSA clusters are also highlighted: ▭, lineage 1 (L1); ▭, lineage 2a, and ▭ lineage 2b (L2a and L2b). Tree scale bar indicates 10 allelic difference. The red circular line indicates the average 24 allelic distance cut-off value for evaluating relatedness based on cgMLST results, as suggested by Schürch et al. (2018)

6. Discussion

6.1 Presence and characteristics of pig-associated LA-MRSA in Hungary

Hungary used to be a country characterised by a low prevalence of MRSA according to a survey conducted by the EFSA in 2008 (EFSA, 2009), as MRSA could be detected in none of the 40 breeding herds examined. Similarly, only 3 out of the 141 Hungarian production farms were positive (2.1%). The present study included a total of 40 pig farms in Hungary. The majority of these were production farms (n = 36), two were pig fattening operations, and only two farms corresponded to the 'breeding farm' category as defined by the EFSA (2009). This is why it was not possible for us to conduct an analysis according to the categories used in the 2008 survey. As the composition of this sample is characterised by the predominance of commercial pig producing farms, the indices of this latter category are also considered.

Compared with the data obtained in 2008, there had been a dramatic increase in prevalence: MRSA could be detected in 82% of the pig herds rearing sows. Based on a 10-year perspective, this trend could have been expected on the basis of studies performed in other countries a few years after the European baseline survey. Between 2008 and 2012, the prevalence increased from 2.1%–3.4% to 23.6% in Poland and from 35.9%–40.0% to 65.5% in Belgium, and this was accompanied by an increase in the genetic variability of the identified strains (Mroczkowska et al., 2017; Peeters et al., 2015). In the case of Poland, this phenomenon was explained by the increasing proportion of breeding pigs imported from countries characterised by a high prevalence of MRSA, including Germany and the Netherlands. In 2012, Denmark also belonged to that category, with an increase in the positive herds of 0.0%–3.5% to nearly 70% in 2014; however, in Denmark the population structure of the strains seemingly became more uniform, as opposed to the examples cited above. Based on the genomic analysis of strains originating from that period and the retrospective study of animal transports, the pyramidal structure of the Danish pig industry and the one-way movement of animals within that structure proved to be the most important factors (Sieber et al., 2018).

In Hungary, the imports of breeding pigs have continuously increased since the 2010s, and structural changes have occurred in the pig industry (Supplementary figure S1). These factors may have played a role in the wide dissemination of MRSA. Seven of the sampled 40 pig herds studied in Hungary belonged to an integration, Holding-1, which besides using imported breeding genetics of Danish origin (Genetics A) has applied the Danish pyramidal structure. The movement of animals was hierarchically organised within the holding, and at the time of the survey, it supplied a further six sampled farms with breeding animal replacements. Other herds had used two types of genetics of Dutch origin, but our sample also included farms working with

hybrid genetics produced in Hungary (Farms 24, 38, and 39), as well as other herds using miscellaneous breeding genetics.

The low number of Hungarian isolates (n = 3) included in the 2008 EFSA survey does not allow us to draw conclusions regarding the genetic variability of the Hungarian MRSA population typical of that time. The genetic characteristics of the MRSA strains isolated in the current study, however, indicate the exclusive spread of strains belonging to the CC398 lineage, as seen in other European countries (Avberšek et al., 2021; Kittl et al., 2020; Mroczkowska et al., 2017; Pirolo et al., 2020).

Comparison of the whole genomes also pointed to the important role played by the inter-herd trade of animals. The strains of clusters C1–C5 were grossly arranged according to the breeding genetics used in the herds. Clusters C1 and C2 mostly included the isolates obtained from Holding-1 working with Genetics A and from the farms supplied by it. The strains of the two clusters showed numerous similarities in both their core genomes and their resistance set encoded by their accessory genomes. Moreover, the inclusion of Danish LA-MRSA sequence data revealed a close phylogenetic relationship between more selected Danish L1 and L3 strains (Sieber et al., 2018) and the Hungarian C1 and C2 isolates, respectively. In the light of these, it is reasonable to suppose that the conditions of the integration can be considered largely a Hungarian reflection of the Danish example, accompanied by the circulation of LA-MRSA strains of Danish import pig origin.

Researchers obtained similar results by comparing MRSA isolates from Southern Italian pig farms with genomic data originating from the prior Danish survey as well as from a study analysing samples from several European countries (Price et al., 2012). Based on the analysis of the whole genomes, the strains isolated from pig farms rearing animals imported from Denmark could be assigned to one of the two dominant clusters (L1 and L3) of the Danish MRSA strains. In addition, the Danish and Italian strains also showed many similarities in their resistance profiles (Pirolo et al., 2020). There were similar patterns in the case of other smaller groups of Hungarian farms, further supporting the importance of animal movements between herds regarding the spread of MRSA.

Of note, there were some exceptions to the above trend. Based on their isolates, some farms working with Genetics A were also included in main genetic Group 2, and vice versa; MRSA strains belonging to C1 were isolated also from farms using other breeding genetics. Based on earlier experience, in rare cases, the simultaneous presence of multiple types of CC398 clonal lines within the same pig farm, also occurs (Avberšek et al., 2021; Fetsch et al., 2016). In the case of the samples evaluated in this study, due to the divergent phenotype of the bacterial colonies, their parallel testing seemed to be justified. However, from most of the samples only one bacterial colony was selected for further study, an approach that prevented the identification of variants that could be genetically very different but with similar phenotypes present in the culture. Such genetically

different lines may be introduced to pig farms not only with carrier pigs, but also with dust, by infected rodents or insects, as well as by colonised humans (Butaye et al., 2016).

Working with livestock substantially increases the risk of colonisation and developing a clinically apparent infection (Fetsch et al., 2021). People working in pig operations are at a markedly high risk (Lewis et al., 2008). Although humans seem to carry MRSA of animal origin only transiently (Angen et al., 2018), other studies, conducted on pig farms, indicate that permanent colonisation is common among farm workers (Köck et al., 2012). In the present study, two thirds of the professionals working on pig farms, a total of 18 people, carried a LA-MRSA CC398 strain. In half of the cases the pig farm was clearly identifiable as the source of colonisation, based on the high degree of similarity between the strains (0–4.5 cgMLST allelic difference and 26–72 SNPs). In the remaining cases, the farm was either negative or a genetically different isolate was found. As at the time of the study the veterinarians involved were also attending other pig farms or other livestock not included in the study, the human strains differing from the farm strains or those isolated from farms with a negative status may have originated from other sources (Walter et al., 2016). Similarly – as mentioned before – in the current study the presence of multiple clonal lines not identified on the farms cannot be excluded.

Most of the human clinical samples included in this study are presumably of livestock origin. This seems to be supported by the fact that the isolates were mostly derived from the major pig-producing regions of Hungary. Although no further data of the patients supplying the samples, including their occupation, are known, a high animal density at the place of residence is a known risk factor for the human population that is not associated with animal production (Anker et al., 2018). During the genetic comparison, these isolates were largely mixed with strains of pig origin, and in most cases a direct spread of pig farm origin could be supposed based on the high degree of similarity found in the cgMLST and SNP distance matrices and resistance profiles. This view is consistent with the results of other studies where the majority of LA-MRSA strains isolated from clinical cases may have been introduced into human health care directly from animal production, retaining their major characteristics (Avberšek et al., 2021; Sieber et al., 2019). Except for two isolates, all clinical isolates in this study showed a high degree of resistance and also carried resistance genes against active compounds used almost exclusively in livestock, like tiamulin and phenicols.

The host and ecological niche adaptation of MRSA CC398 is accompanied by the gain and loss of mobile genetic elements, like the human-associated IEC (Sieber et al., 2019). Although regaining the IEC seems not to be a prerequisite for the pathogen’s survival or pathogenicity in the human host, its appearance in LA-MRSA strains is an indicator of the spillover to

humans, according to the latest research (Matuszewska et al., 2022). Four of the strains in this study carried the IEC, but only one of them (N19281) showed a closer relationship with the strains of pig origin. Due to the low number of samples and the lack of metadata, no sound conclusions can be made regarding the microevolutionary changes of the Hungarian LA-MRSA population. However, the emergence of IEC-carrying LA-MRSA isolates call for further studies to assess the possibility of human-to-human spread and the risk posed by such a scenario to public health in Hungary.

Even if the matter of host adaptation is somewhat controversial, the high level of antibiotic resistance of LA-MRSA is already a public health concern, as half of the strains isolated from pig farms showed resistance or a non-wild-type phenotype to at least six tested active compounds besides beta-lactams. Of the encoded resistance determinants, the multi-drug resistance (MDR) genes that provide resistance to multiple antimicrobial agents cause the greatest concern. Almost all MDR genes of this study are known to be coded on mobile genetic elements and have been previously detected in staphylococci of both human and animal origin (Schwarz et al., 2018).

The plasmid-borne *cfr* gene identified in a pig farm isolate and in a human clinical isolate in this study deserves particular attention, as it encodes resistance to five classes of antimicrobials at the same time (the PhLOPSA resistance pattern) (Schwarz et al., 2018). Of the compounds involved, the oxazolidinone derivative linezolid is especially important in human medicine, as it is one of the antibiotics that can be used against MDR Gram-positive bacteria and primarily against MRSA (Kloss et al., 1999). The importance of linezolid resistance is underlined by the fact that MRSA, other *Staphylococcus* spp., and Enterococcus spp. strains carrying the cfr gene can cause nosocomial infections (Lazaris et al., 2017; Mendes et al., 2013; Morales et al., 2010). Despite its importance, however, it is reassuring that the data obtained in the years since the first detection of the cfr gene do not suggest an epidemic-like spread of this resistance gene (Wang et al., 2015; Witte et al., 2011). At the same time, pleuromutilins and florfenicol widely used in animal production exert high selection pressure for the enrichment of this resistance gene not only in the case of MRSA strains, but also in other species. Therefore, the detection of this resistance gene in Hungary and the presence of cfr-positive MRSA calls attention again to the increased public health risk posed by the high-level MRSA carriage of people working in the pig industry (Sieber et al., 2019).

The present study has some limitations. On the one hand, factors influencing the risk of MRSA occurrence on a pig farm are not completely known; hence, appropriate stratified random sampling was not possible. Although the sampled farms were randomly selected to eliminate some bias in sampling, the results were not intended to be representative for the Hungarian

swine population. On the other hand, only 38 of the hundred selected farms took part in the study, less than the number needed for a proper estimate (64 farms). The low response rate further underlines that the present prevalence estimate might deviate from the (yet unknown) true prevalence of MRSA-infected Hungarian swine farms. It is to be noted, however, that the results may adequately reflect the conditions of the important pig-producing regions of the country (Figure 1). In addition, when evaluating the obtained prevalence data, the fact that the method of sampling may reduce the sensitivity of the test must be considered. In a preliminary study, the EFSA found that the method based on testing dust samples, used to survey the infection status of pig farms, was somewhat less reliable than simultaneously sampling 60 pigs (EFSA, 2007). Therefore, when evaluating the results of the baseline survey conducted in 2008, the EFSA called attention to the fact that the actual prevalence was presumably higher than that suggested by the obtained data, even in areas showing an apparently low prevalence (EFSA, 2009). Taking the above facts into account, the number of infected farms in the present study may be lower than the actual number. This seems to be supported by the fact that pigs from some farms found to be negative during the study subsequently yielded MRSA strains in further routine diagnostic investigations (data not shown). Nonetheless, due to its easy and rapid implementation, the collection of environmental dust samples is a widespread method for the detection of MRSA in animal populations, in most cases supplemented by the collection of samples from animals (Geenen et al., 2013; Mroczkowska et al., 2017; A. Schnitt et al., 2020).

In conclusion, the results of this study showed that livestock-associated MRSA has become extremely prevalent in Hungarian pig farming. In accordance with other findings from then-low prevalence countries, like Denmark or Poland, both massive live animal importing and inter-herd movement of positive animals could contribute to its rapid spread. Besides being abundant, most strains presented a wide range of antimicrobial resistance and the capacity to colonise and infect humans. Hence, the role of LA-MRSA as a zoonotic pathogen should also be re-evaluated and its epidemiology regularly monitored in Hungary.

6.2 MRSA strains of bovine milk origin in Hungary

According to the investigation of 626 individual isolates originating from 42 dairy holdings, the occurrence of methicillin resistant strains seems to be rare among SA isolates from bovine milk. The results suggest prevalence rates similar to that of the last surveys conducted in Hungary. Between 2002–2003 Kaszanyitzky and colleagues detected only five MRSA strains among 867 bovine milk originated SA isolates (0.58%) (Kaszanyitzky et al., 2004). In another study, no MRSA could be isolated from bulk tank milk samples of 20 dairy farms (Peles et al., 2007).

As a comparison, the 42 holdings in our survey represent approximately 7–8% of the total number of performance-tested dairy holdings, according to the register of LPT Ltd. The number varied between 519 and 527 during the survey period (data available at https://www.atkft.hu/partnertajekoztato-hirlevel/). As no further statistics could be performed on our data, it can only be assumed that the burden presented by MRSA is still not significant in the Hungarian dairy sector. This tendency is in accordance with data from other European countries, where the overall prevalence of MRSA-positive dairy herds varies between 0–10% (Arne Schnitt et al., 2019). Meanwhile a large-scale meta-analysis of the timeframe 2007–2016 also implies a low worldwide prevalence of 2.2% (Ou et al., 2017). The percentage of MRSA within SA isolates of milk is also comparable with the observations of others (Luini et al., 2015).

In this study, most of the isolates (20/31) belonged to the ST1–t127 genotype. This genotype has emerged as a human community associated MRSA in the USA carrying the potent human virulence factor PVL (encoded by the *lukS-PV/lukF-PV* gene); it has since been reported worldwide with variants being either PVL-positive or PVL-negative. The PVL-negative variant of the lineage from animals and a dairy worker was first described in Hungary in the early 2000s by Juhász-Kaszanyitzky and co-workers (2007), who determined the genotype but did not investigate the resistance profile of the isolates. More recently, the lineage is widely distributed in Italy and occasionally reported from other countries of South-Eastern Europe, both from humans and animals (Cuny et al., 2015c). In dairy herds, ST1–t127 MRSA is frequently recovered from subclinical and clinical mastitis cases. Phylogenetic studies suggest a potential human origin of the cow-related strains, while the clonal lineage retains specific virulence and immunomodulatory genes, and thus is able to colonise the human host (Alba et al., 2015).

The second most frequent clonal lineage in this study was clonal complex (CC) 398. This initially swine related MRSA genotype has also appeared and become widely distributed in most domesticated and wild animal species and also in humans, due to its low host specificity (Cuny et al., 2015c). It may cause subclinical and eventually clinical mastitis outbreaks in cattle herds (Spohr et al., 2011; Vanderhaeghen et al., 2010), yet the pathogenic ability and role of CC398 in dairy production still needs further investigation. Regardless of their origin and virulence genes, all CC398 strains can colonise the human host (Cuny et al., 2015c).

Three strains shared the same ST22–t032 genotype, which is identical to the human epidemic UK-EMRSA-15 clonal lineage. The lineage was proven to colonise horses and companion animals (Vincze et al., 2014; Walther et al., 2012). MRSA CC22 has also been implicated in bovine mastitis and simultaneous human nasal carriage on one farm in Italy. The authors concluded that humanosis was the only possible way of introducing the pathogen into the closed dairy herd (Magro et al., 2018). The newly described ST5982–t458 belonged to CC97, which was originally described in ruminants, either as methicillin-sensitive SA or MRSA, and accounted as one of the most prevalent genotypes. Some sub-lineages of the clonal complex have become pandemic within the human population in recent years and could already be isolated in countries of four continents (Spoor et al., 2013).

In the case of MRSA, antibiotic pressure is a major driver for selection. All but two strains were resistant to at least three or more classes of antimicrobial agents, fulfilling the term of multiresistance. The antibiogram also helps to allocate the recovered isolates to the most probable host group. For instance, tetracycline resistance is a hallmark of more LA-MRSA lineages, due to the long term and wide scale usage of the compound in farm animal husbandry (Price et al., 2012). Only three strains of ST22–t032 showed tetracycline susceptibility, further supporting the possible human origin of these latter isolates.

Resistance to ciprofloxacin, clindamycin, kanamycin and streptomycin was also widespread among the investigated isolates. These compounds are categorised as critically important or highly important antibiotics for human medicine, according to the World Health Organisation (2018). The detected genes coding for kanamycin, gentamicin, tobramycin, tetracycline and streptomycin resistance are encoded on mobile genetic elements (i.e., plasmids and transposons), allowing horizontal gene flow among strains or even species of staphylococci in different hosts. This highlights the possibility of a mutual exchange of resistance determinants between staphylococci of human and animal origin (Wendlandt et al., 2013).

6.3 Presence of MRSA at equine clinics and in the horse population outside equine clinics in Hungary

The presence of MRSA in North American equine clinics has been known since the 1990s (Seguin et al., 1999), while the first documented European cases may be put at the middle or end of the 2000s (Cuny et al., 2008; van Duijkeren et al., 2008). During the years that have elapsed since then, the emergence of the pathogen was reported from numerous large horse clinics of Europe (Islam et al., 2017; Sieber et al., 2011; A. Van den Eede et al., 2009; Vincze, Brandenburg, et al., 2014). The MRSA-positive cases of the Department and Clinic of Equine Medicine (DCEM) of the University of Veterinary Medicine Budapest were elucidated by two successive retrospective studies. According to the results of these two studies, this pathogen caused infections among the patients of the DCEM every year since at least 2009. From 2013, the case number of 2–5 confirmed cases per year drastically increased, and MRSA continuously caused infections until December 2018 except for a few months (Supplementary Figure S3). The colonisation and permanent presence of MRSA are not unusual in equine clinics (Sieber et al., 2011; Weese et al., 2004); however, to the best of our knowledge this study is the first to investigate the MRSA isolates of an equine clinic and their evolution in such a long-time span.

The strains isolated at the DCEM belonged to the clonal lineage typical of equine hospitals. Their common characteristics include the t011 *spa* type, the SCC$_{mec}$IV cassette type, gentamicin resistance coded by the *aac(6')Ie-aph(2")Ia* gene (Cuny et al., 2008; van Duijkeren et al., 2010), and the carriage of a point mutation (canSNP1748T) specific only for this sub-clone (Abdelbary et al., 2014). In addition to their genotype, the extensive resistance profile of these strains was also extremely uniform: besides beta-lactam and aminoglycoside antibiotics, all strains were also resistant to tetracyclines and potentiated sulphonamides and the majority of them to streptomycin and fluoroquinolones, too. Over time, a certain proportion of the strains became resistant to further antimicrobial agents (chloramphenicol and rifampicin). Based on the highly similar MLVA profile of the above strains and the isolates obtained between 2011 and 2016 it could be assumed that the strains isolated at the DCEM in that period constituted a single clonal lineage, which assumption was confirmed by the whole-genome comparison of the representative strains. At the same time, the three strains originating from a period before 2011 were clearly distinguishable from the former strains but very similar to one another.

At the DCEM we identified several factors which were considered to be risk factors for long-term persistence in previous studies. On the one hand, MRSA can remain viable in the environment, especially in the dust of stables, for a long time, continuously contaminating the skin and airways of the incoming equine patients without causing an actual epizootic (A. Van den Eede et al., 2012). During the environmental survey conducted in 2017, we successfully

cultured the same clone from all stables and also from the dust in other units of the clinic. Further difficulties in reducing the environmental pathogen load may be due to the fittings and equipment of the clinical premises that are difficult to clean and disinfect, such as the porous cover of surfaces, the drinkers, and the hay racks. In addition to the usual fittings, in the stables and treatment rooms of the DCEM the numerous wooden surfaces and the brick floor covering of the boxes provide a suitable surface for the survival of the bacterium (Weese et al., 2004b; Humphreys et al., 2009).

Another important risk factor facilitating the permanent presence of MRSA is the human staff working with horses, which may participate in spreading the pathogen partly as a mechanical vector through the transmission of contaminated dust and partly through their colonisation by the bacterium (van Duijkeren et al., 2010; Sieber et al., 2011). During the study, eight out of the 36 staff members of the DCEM (22.2%) were found to carry a strain identical with the resident clone in almost all its properties. In similar surveys, this ratio ranged between 9.4% and 22.2% (Sieber et al., 2011; van Duijkeren et al., 2010). Among the staff members of the DCEM, primarily the horse carers and the internists were carriers, while all the surgeons were negative. This is consistent with the observations made at equine clinics and in human healthcare that the carriage of MRSA is more common among people who have daily connections with infected horses or patients and work in their potentially contaminated environment (Dulon et al., 2014; van Duijkeren et al., 2010). Thorough hand hygiene seems to be a key factor in preventing the mechanical transmission of MRSA. The use of disposable gloves, regular handwashing and disinfection between successive patients can substantially decrease the occurrence of cases as MRSA becomes resident in a healthcare institution (Humphreys et al., 2009; Muto et al., 2003; Sieber et al., 2011). Non-compliance with the strict hygienic rules may also have contributed to the recurrence of outbreaks and the long-term persistence of MRSA infection at the clinic.

Incoming horses carrying the pathogen may also contribute to maintaining MRSA infection in a clinic, and they may even introduce new genotypes (Bortolami et al., 2017). Therefore, it seemed to be logical to determine the carriage rate of MRSA strains in horses referred to the clinic, and the detailed analysis of the isolated strains was also performed. During the eight months of the study only 8 of the horses sampled on arrival at the clinic (6%) proved to be positive. This value is slightly lower than the result of similar studies performed elsewhere in Europe (9–11%) (A. Van den Eede et al., 2009; van Duijkeren et al., 2010). Interestingly, the majority of the identified strains (n = 7) showed a high degree of similarity to the clonal lineage present in the clinic both in their resistance profile and their cgMLST alleles (Figure 7). One strain represented a new genotype (ST1–t127–SCC$_{mec}$IV), which had been present at the university equine clinics of Vienna and Bern for years (Cuny et al., 2008; Sieber et al., 2011),

but seems to be rare outside equine hospitals. In a relevant German study one strain of such genotype (0.4%) was isolated when characterising 272 *S. aureus* isolates of equine origin in the period between 2011 and 2015 (Cuny et al., 2015b). The strain collection of the DCEM did not contain such a strain, the significance of which is probably negligible in the Hungarian horse population.

Regarding the MRSA screening of hospitalised horses, Van den Eede remarks that the horses referred to hospitals do not necessarily reflect the MRSA carriage patterns of the healthy horse population (Van den Eede et al., 2012), which assumption is supported by the results of another case-control study as well (Weese et al., 2007). To estimate the carriage rate and to balance the sample, we conducted screening tests in several regions of Hungary, using nasal swab samples from 325 asymptomatic horses of 24 horse studs. From these samples we could not culture MRSA. The above-cited Dutch study conducted in 2007 brought similar results: only one out of 189 horses from 10 horse farms (0.5%) was found to carry MRSA which proved to be of the CC398–t011–SCC*mec*V type (Van den Eede et al., 2012). A German study found that 2 out of 223 horses were carrying MRSA, the EHA strain in both cases (Kaspar et al., 2019). A Danish survey demonstrated a slightly higher prevalence (4.2%). Ten out of the 401 sampled horses (2.5%) were carrying the EHA-MRSA strain, while the remaining seven isolates represented a different genotype (Islam et al., 2017). From the results obtained in horses referred to hospitals and those examined in riding establishments it appears that the Hungarian horse population has a rather low carriage rate of MRSA and mostly carries strains identical with the hospital-associated clone.

Equine hospitals serve as a possible source of contamination, the probability of which is confirmed by examination of the strain collection of DUO-BAKT laboratory. A classical epidemiological connection with one of the equine clinics could be found in the case of 7 out of the 12 EHA-MRSA positive horses sampled in private practices: these horses had been hospitalised or treated as out-patients of one of the equine clinics or possibly had come into contact with horses treated at an equine clinic (Table 3). Hospitalised equine patients may have a rather high level of MRSA contamination. Of the 116 horses tested at the time of discharge from the DCEM, 54 (46.6%) gave an MRSA-positive sample, which alone poses a substantial risk for the horse population outside the clinic. Van Duijkeren and colleagues also obtained similar results: 42% of the hospitalised horses were MRSA positive at least once during their stay in the hospital (van Duijkeren et al., 2010). It is important to point out that only 41 horses were sampled at the time of both their arrival and their discharge, and thus had a known MRSA status on arrival. In that smaller sample, the rate of MRSA carriage on discharge was around 30%, and 4 out of those horses (10%) had arrived as a carrier.

Further conclusions regarding MRSA-positive horses discharged from the hospital are prevented by the fact that neither the chance of colonisation after MRSA contamination nor the time needed for elimination of the pathogen are known. Therefore, the risk posed by horses of unknown MRSA status and returned to their original environment without a quarantine cannot be estimated in a reliable manner (van Duijkeren et al., 2010). No data are available on MRSA contamination yet. The two studies investigating the duration of MRSA carriage after infection report a wide time frame: 2 to 23 months, on average 5 months (Larsson et al., 2011; Weese et al., 2005b), which seems to be an extremely long time in the practice for keeping a recovered horse in quarantine. At the same time, the studies performed on pigs and humans coming into contact with pig farms indicate that only a small percentage of pigs and humans exposed to a contaminated environment, primarily with dust, become real carriers, while in the majority of pigs and humans MRSA disappears from the nasal mucosa within a short time (Angen et al., 2018; Bangerter et al., 2016). The latter assumption is supported by the observation that among the horses screened in horse studs there were 14 animals which, in the year before the sampling, had been treated at an equine clinic (DCEM and Clinic 2, Vienna) known to be MRSA positive, and all of them produced negative samples during the testing. However, it is important to emphasise that a high positivity rate at the time of discharge increases the chances of colonisation; therefore, it seems to be important to clarify the issues regarding elimination of the pathogen as soon as possible.

Although MRSA strains may circulate also in the Hungarian horse population, the nosocomial nature of these infections is confirmed also by the detailed study of the conserved MRSA strains of another equine clinic and the experience gained from an actual epizootic associated with that clinic. Clinic 2 is an animal hospital of lower patient turnover, compared to the DCEM, offering services for horses and companion animals. MRSA was first detected in 2008, and until 2010 several outbreaks occurred at the clinic. Another disease outbreak was observed in 2013. The whole-genome and resistance profiles of the strains of equine origin isolated from the outbreaks showed a high degree of similarity to isolates obtained at the DCEM and at external equine practices.

The 2018 outbreak was investigated by sampling the clinic and three horse studs involved. At the time of sampling, two weeks after the first case but before cleaning and disinfection Clinic 2 was still heavily infected. Samples from all equine patients staying in the clinic (n = 5) and those from 26% of the staff were EHA-MRSA positive. Of the humans found to be infected, two veterinarians did not work directly at the equine division. Their positive status may be explained by the substantial contamination of the environment also in this case. Sixty per cent of the swab samples were also positive, and no MRSA-free areas were found among the premises examined, including those dedicated for small animals and other servicing rooms.

Interestingly, the strains were of the same genotype but could be divided into two characteristic phenotype groups based on their resistance profile. There were no relevant differences regarding their occurrence, as the strains of both types could be cultured from samples taken from the horses, the staff and from the environment too.

Surprisingly, two of the horse studs (S1 and S3) had no MRSA-positive animals except the one that had passed through the infection, even though the actual infection was revealed only about a week after the affected horses had arrived home. Moreover, the stable containing the index patient found heavily contaminated. In addition to the box of the affected horse, the pathogen could be detected in other areas of the stable as well, and two out of the ten sampled staff members proved to be EHA-MRSA carriers. Again, this indicates that MRSA does not spread readily among horses outside equine hospitals, and only the permanent contamination of the environment presents a high risk of infection. This is not contradicted by the fact that in the third horse stud (S2), besides the two horses discharged from Clinic 2, two further, asymptomatic horses were carrying a similar EHA-MRSA strain. These two animals had not been at a clinic earlier, nor could they come into contact with MRSA-positive horses placed into quarantine after their arrival, and thus the source of their contamination is uncertain.

According to our initial assumption, the outbreak in the stud sampled in 2019 was independent of the previous cases, in view of the elapsed time and the lack of contact with the equine clinic. The only classical epidemiological connection was the attending veterinarian, who provided several positive samples in both 2018 and during the current outbreak but underwent a successful decolonisation therapy after the first outbreak. In addition to the veterinarian, several employees of the stud, their family members and some of the horses were also asymptomatic carriers. At the same time, regarding the MLS_B resistance mediated by the *erm*(C) gene a certain proportion of the detected EHA-MRSA strains differed from the isolates of the 2018 outbreak and the isolates obtained from the DCEM in the same period only in a few cgMLST alleles (Figure 7). It should be mentioned that we did not find the *erm*(C) resistance gene in the tested isolates originating from the same period but from other sources (DCEM, Clinic 2, horse studs) either. The appearance of the resistance gene in the population may be explained by the routine use of macrolide antibiotics against *Rhodococcus equi* infection, as reported by the veterinarian. In that case, the genotype of the strains, the extensive carriage and the appearance of the new resistance gene may indicate that this genetic line may have been circulating in the population for a long time prior to the outbreak.

The whole-genome comparison and detailed analysis of the EHA-MRSA strains identified during our studies showed a high-level homogeneity of the strains isolated in the same period, irrespective of their place of origin. The strains of the two identified clusters (allelic difference <24)

of the similarity tree also separated according to the time of isolation. This separation is even more conspicuous when comparing the Hungarian and the other European strains. Within the clusters, the clinical isolates of the horse studs and the MRSA strains of horses arriving at the DCEM were mixed with the strains originating from outbreaks at the two sampled equine clinics. The appearance of certain traits of the strains also seemed to change with time, and this was confirmed by the analysis of the strain collection of the DCEM, performed in order to get a higher resolution. Between 2008 and 2010, the presence of strains of the L1 cluster appears to be exclusive. Strains of the L2 cluster first appear in 2011, then from 2012 exclusively L2 strains are found, presumably fully replacing the L1 strains. The first strains carrying an ϕSaeq1-like prophage and horse-specific virulence genes encoded on it appeared in 2013. These *lukPQ*-positive strains formed a new sub-cluster within L2, also almost completely outplacing the previously isolated strains of L2. Interestingly, the number of MRSA-positive cases identified at the DCEM also started to increase from that year. The fitness-increasing effect of horse-specific virulence genes can only be inferred from *in vitro* experiments studying the efficiency of virulence factors (de Jong et al., 2018; Koop et al., 2017) and from the fact that the prophage carrying these genes is widespread in horse-associated *S. aureus* strains while it is mostly missing from the strains isolated at equine hospitals (de Jong et al., 2018; Koop et al., 2017). Thus, its appearance in EHA-MRSA lineages indicates adaptation to the host species (Walther et al., 2018).

The increased case number and the extensive resistance of MRSA made it necessary to introduce new antimicrobial agents in the DCEM. The combination of chloramphenicol and rifampicin was added to the treatment protocol in October 2014. Resistance to these two antimicrobial agents among the isolates became widespread after 4 and 11 months, respectively, probably due to the massive selection pressure posed by the antibiotics (K. Kadlec et al., 2012; Schwarz et al., 2014). While the occurrence of chloramphenicol resistance decreased, presumably as a result of more targeted treatments, the rifampicin resistance encoded in the bacterial chromosome became fixed in the clonal lineage. Also, because of the MRSA infections and an intercurrent viral outbreak, the previously used disinfection with bleach (sodium hypochlorite) was replaced by a product containing benzalkonium chloride. Subsequently, the gene encoding resistance to quaternary ammonium salts appeared at the beginning of 2015 (Bjorland et al., 2003), to become widespread after the viral outbreak from the end of 2015, almost exclusively in the isolates of the DCEM. Although the efficiency of mediated resistance is disputed (Jennings et al., 2017), its emergence can also be regarded as a new trait appearing as a result of the selection pressure.

The new traits that appeared during the evolution of the L2 clonal lineage, thus the virulence and resistance genes, occur sporadically also among the European strains included in the

study, which supports the indigenous nature of the changes that have occurred. A similar conclusion can be drawn when studying the prevalence of the individual traits. Although only few studies have dealt with this question, the ϕSaeq1-like prophage has been described only from German EHA-MRSA isolates so far (Walther et al., 2018); these strains were included also in the comparison performed in this study. Similarly, few studies have included chloramphenicol and rifampicin in the antimicrobial susceptibility testing of MRSA strains of equine origin; however, these studies report the sporadic occurrence of resistance (Guérin et al., 2017).

Our results indicate the gradual adaptive micro-evolution of the clonal lineage taking place presumably in only a few epicentres, as well as its spread from the epicentres into the horse population. Such epicentres may include, besides the equine clinics, certain intensive horse-keeping establishments, e.g. studs, where the conditions required for enrichment of the pathogen are present. Risk factors may include high animal density, the frequent contact of horses with veterinarians, regular antibiotic treatments, high rotation of horses and contact with a permanent group of the clinical or horse stud staff who simultaneously treat affected horses as well (Weese et al., 2007; van Duijkeren et al., 2010). Therefore, a complex strategy focusing on the epicentres should be elaborated to control the MRSA infections of horses. The emphasis is on the preventive measures, first of all on the adequate hygiene (Weese et al., 2005b). In addition to the regular, systematic cleaning and disinfection of the contaminated stables, this must include checking the efficiency of such operations as well. Providing the staff and, in teaching clinics, the students with personal protective equipment is at least as important. The mechanical transmission of contaminated dust can be decreased by thorough hand hygiene and by changing the protective clothing between stables. In addition to this, the results of both the current study and the investigations of other authors also show that EHA-MRSA can colonise the skin and nasal cavity of humans coming into contact with the carrier horses and their contaminated environment (Cuny et al., 2017).

Both at the clinics studied by us and in the MRSA-infected horse farms a significant proportion of the staff members were carrying the pathogen. However, according to the results of an earlier German study (Cuny et al., 2015b) it can be assumed that the infections caused by EHA-MRSA strains occur sporadically also in Hungary and do not pose a real risk to people dealing with horses at present. At the same time, the high-level EHA-MRSA carriage of veterinarians presents a concern for equine medicine and should receive more attention because of the possibility of human-to-animal spread (Koop, 2016). The probability of human colonisation is markedly decreased by using dust masks. Under pig farm conditions, only 9% of humans wearing a mask and 62% of those not wearing one proved to be MRSA positive on leaving the farm. All of the samples taken from the former group in the subsequent days

became negative, which shows the low germ count taken up and the rapid elimination of the bacterium (Angen et al., 2018). Due to the measures taken because of the COVID-19 pandemic, wearing a mask became a routine practice at the DCEM. Although the effects of this were not studied, the subjective experiences are favourable. Using the preventive measures outlined above, MRSA contamination of the equine clinics and the endemically infected horse-keeping places can be reduced drastically, and at the same time the dissemination of the pathogen into the horse population can also be substantially decreased. The use of adequate hygiene practices may also help decrease the importance of the eradication therapy of colonised horses and humans. In addition to posing a burden on the treated humans and animals, medicinal eradication has uncertain outcome in horses (Weese et al., 2005b), while in humans the probability of reinfection is high (Sieber et al., 2011).

Finally, we have to mention the limitations of the studies as well. First, it is important to clarify the reason for performing the two retrospective investigations. On the first occasion, the *Staphylococcus* strains were selected for further study exclusively on the basis of the strain collection records, primarily the presence or absence of haemolysis, which proved to be an unfortunate decision for several reasons. On the one hand, as it turned out later, for numerous haemolytic strains the data regarding haemolysis were not included in the conservation logbook. Thus, these strains were left out from the first examination. On the other hand, the entries of the conservation logbook itself proved to be incomplete, and thus several *Staphylococcus* sp. strains were not named on the species level either, already in the pre-screening phase. These were selected on the basis of the diagnostic reports and tested in the second study. Based on its results, the second study only extended our knowledge regarding the presence of the MRSA clonal lineage. In other respects, it did not modify substantially the conclusions of the first study, i.e. that since 2011 the MRSA infections at the DCEM have been caused by a population showing high clonality, which has gradually adapted to the ecological niche: to the environment of the clinic and to the host species.

During the study of horse-associated MRSA the evaluation of genetic relationships among the strains proved to be one of the greatest difficulties, partly due to the number of samples tested and partly because of the method applied. To establish the epidemiological relatedness of two strains originating from different places, the variability of the bacterial population in the two places and the resolving power of the method used for differentiation must be known. If the difference between the two strains (in the present case the allelic difference) does not exceed the degree of variability found within the two populations, the two strains can be regarded as linked (Schürch et al., 2018). From the outbreak at Clinic 2 we could sequence only few strains from a given place, and therefore the variability of the population and the relatedness of strains to other isolates of different origin could not be evaluated in a reliable manner.

Besides its extremely high resolving power, the core-genome and maximum common genome MLST approach used for the comparison of strains has its limitations. This method compares the allele variants of gene loci and differentiates the strains from one another on that basis. As a result of this, it takes into account the mutations of the more variable segments with a lower weight and does not take into account at all the variability of the non-coding segments. Moreover, what gives the stability of the method, at the same time reduces its resolving power. By studying the mostly fixed, slowly changing region of the genome, we disregard the accessory genome changes playing an important role in the rapid adaptation of *S. aureus* (McCarthy et al., 2014), and thus probably missing changes that could increase the resolution and result in a more detailed clustering.

Last but not least, the trees constructed on the basis of the cgMLST reflect the similarity between the strains and not the phylogenetic relationships, and therefore the study of their structure allows only limited conclusions regarding the actual relatedness of the strains. It is important to mention that the applicability of cgMLST was validated mostly for different MLST types of human hospital disease outbreaks, but not for LA-MRSA CC398 strains (Leopold et al., 2014; Jensen et al., 2020). Still, the variability observed among equine MRSA strains in some European countries and the comparison to the domestic swine-related isolates suggest a high homogeneity of the Hungarian equine-related isolates.

For the above reasons, based on the small allelic differences found in the case of the Hungarian strains, the time-dependent variability of the accessory genome-related resistance and virulence genes, and the classical epidemiological data we can only speak about evolution of the clonal lineage interpreted on regional level. Further statements about the events of the epicentres (clinics, horse studs) serving as possible locations of the evolutionary changes and about their epidemiological connections could be made only in possession of a higher number of classical epidemiological data and sequenced isolates, and a more detailed knowledge of the MRSA genome.

7. Overview of the new scientific results

Ad 1. During a wide-scale survey of Hungarian pig farms marked increasing of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) clonal complex (CC) 398 prevalence was reported in comparison to the last similar records from 2008. Characterisation of isolates revealed a high degree of genetic homogeneity of strains and identified several multi-resistance genes transmittable to the human microbiome. Results set a new baseline to which future studies investigating the efficacy of recent antibiotic-related regulations may compare their data.

Ad 2. The high degree of genetic similarity of LA-MRSA strains isolated from clusters of farms rearing the same swine genetics reinforced the role of both domestic and transboundary live animal trade networks in the spread of the pathogen.

Ad 3. A high LA-MRSA carriage rate of swine professionals (veterinarians and farm managers) was observed. Whole genome sequence data indicate the occasional spill-over of LA-MRSA strains of pig origin into the human host. Genetic traits of human clinical specimen derived LA-MRSA isolates indicate possible readaptation of the pathogen to the human host in Hungary.

Ad 4. In the frame of a prospective screening, the low prevalence and heterogenous genetics of bovine milk originated MRSA was confirmed. The results showed a low, yet unchanged prevalence compared to that of the last similar surveys conducted in Hungary. The genetic characteristics of recent and conserved strains revealed the circulation of genotypes with low host specificity and zoonotic potential.

Ad 5. The occurrence and prevalence of equine-related MRSA strains was investigated for the first time in Hungary. Whole genome sequence-based comparison of Hungarian isolates revealed gradual, time-related adaptive microevolution of a highly clonal population throughout the country.

Ad 6. The whole genome sequencing and publication of 106 strains sets a reference collection of Hungarian LA-MRSA CC398 genome data.

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9. Scientific publications

9.1 Scientific publications related to the doctoral thesis

In peer-reviewed journals

Albert, E., Sipos, R., Perreten, V., Tóth, Á., Ungvári, E., Papp, M., Dán, Á., Biksi, I.: **High prevalence of livestock-associated methicillin-resistant *Staphylococcus aureus* in Hungarian pig farms and genomic evidence for the spillover of the pathogen to humans**, Transbound. Emerg. Dis., 2023. Article ID 5540019, 2023.

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Albert, E., Sipos, R., Jánosi, S., Kovács, P., Kenéz, Á., Micsinai, A., Noszály, Z., Biksi, I.: **Occurrence and characterisation of methicillin-resistant *Staphylococcus aureus* isolated from bovine milk in Hungary**, Acta Vet. Hung., 68. 236-241, 2020.

Albert, E., Biksi, I., Német, Z., Csuka, E., Kelemen, B., Morvay, F., Bakos, Z., Bodó, G., Tóth, B., Collaud, A., Rossano, A., Perreten, V.: **Outbreaks of a methicillin-resistant *Staphylococcus aureus* clone ST398-t011 in a Hungarian equine clinic: emergence of rifampicin and chloramphenicol resistance after treatment with these antibiotics**. Microb. Drug Resist., 25. 1219-1226, 2019.

Conference oral presentations

Albert, E.**: Experiences with methicillin-resistant *Staphylococcus aureus* derived from horses in Hungary,** XXVII Annual Conference of the Hungarian Association of Equine Practitioners, Balatonkenese, Hungary, 2019.

Albert, E.: **A multidrug-resistant bacterium in the Hungarian swine sector**, XXVIII. Köves Napok, Egerszalók, Hungary, 2019.

Albert, E., Jánosi, S., Noszály, Z., Sipos., R., Erős, Á., Kelemenová., B., Biksi., I., Szenci, O.: **Methicillin-resistant *Staphylococcus aureus* strains isolated from Hungarian dairy herds in the past 15 years**, XVIII Middle-European Buiatrics Congress, Eger, Hungary, 2018.

9.2 Other scientific publications

In peer-reviewed journals

Albert, E., Sahin-Tóth, J., Horváth, A., Papp, M., Biksi, I., Dobay, O.: **Genomic evidence for direct transmission of *mecC*-MRSA between a horse and its veterinarian**, Antibiotics,12. 408, 2023.

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Petróczki, F. M., Pásztor, Á., Szűcs, K. D., Pál, K., Kardos, G., Albert., E., Horváth, B., Ungvári, E., Béri, B., Peles, F.: **Occurrence and characteristics of *Staphylococcus aureus* in a Hungarian dairy farm during a control program**. Pathogens, 10. 104, 2021.

Német, Z., Albert, E., Dán, Á., Balka, G., Szenes, Á., Sipos, R., Bódizs, S., Biksi, I.: **Genomic analysis of *Staphylococcus aureus* strains originating from Hungarian rabbit farms reinforce the clonal origin of various virulence types**. Animals, 10. 1128, 2020.

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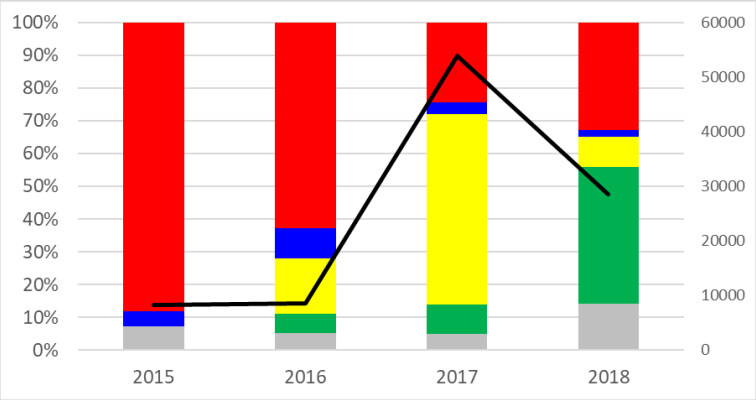
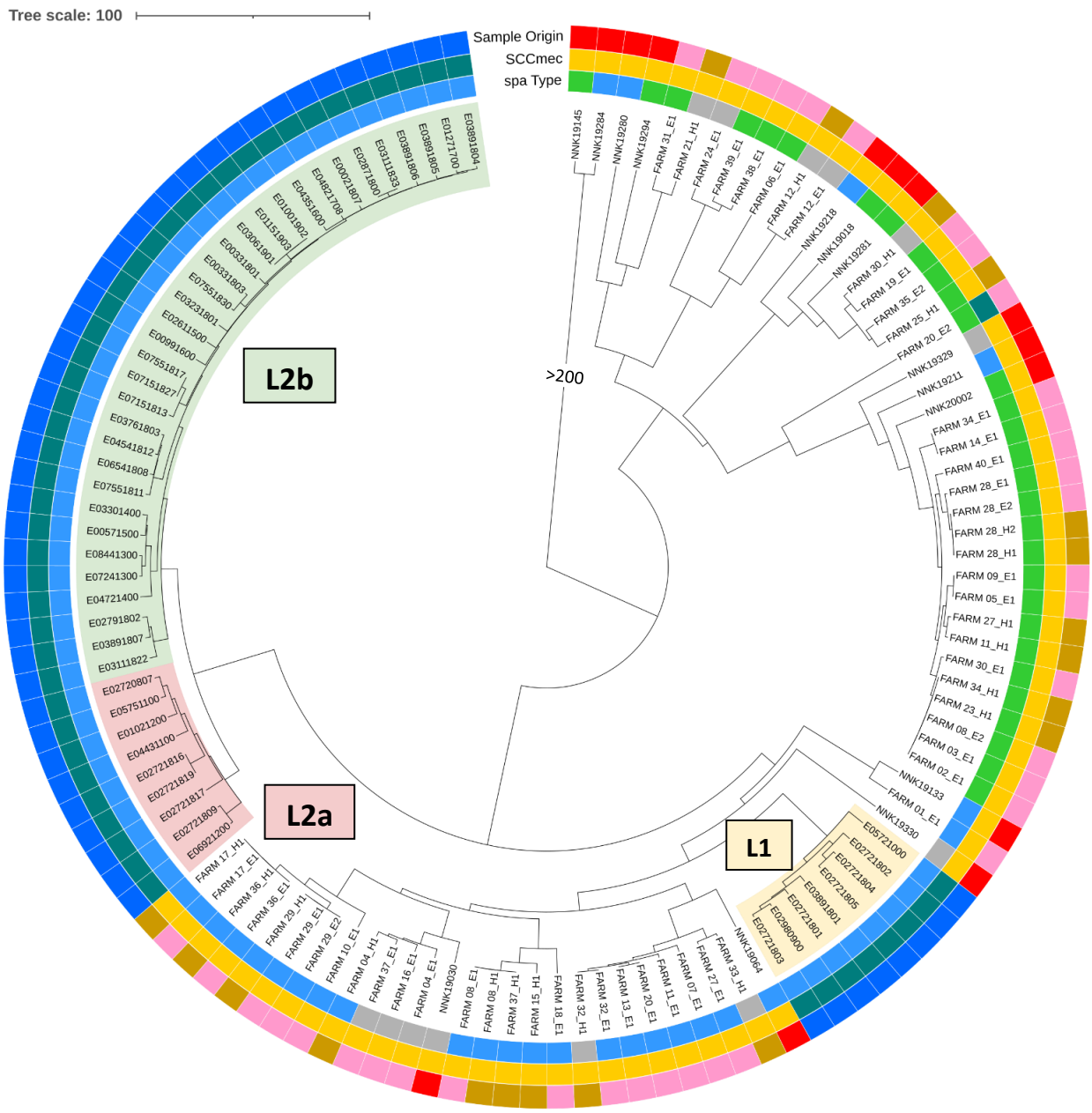
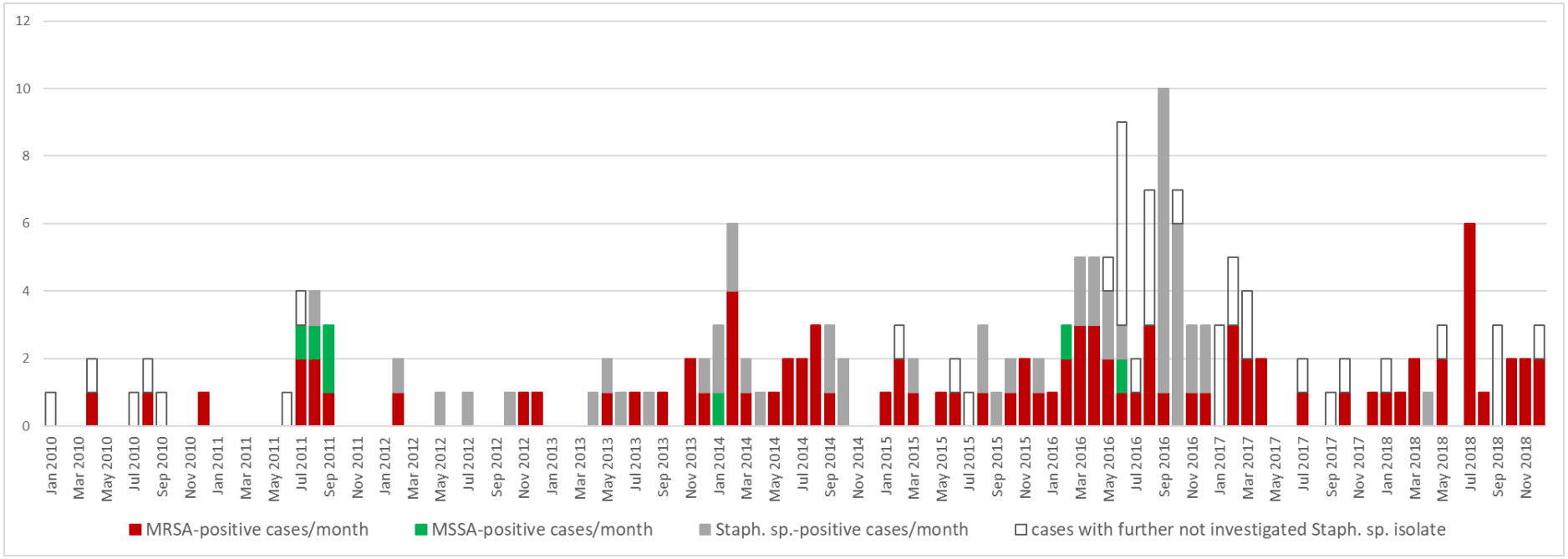
10. Appendix



Supplementary Figure S1 | *Breeding gilt import to Hungary between 2015 – 2019*. Importing countries are colour coded: , Denmark; , France; , Germany; , Czech Republic; , other countries. The total number of imported gilts are shown on the secondary ordinate. Data were extracted from the EU Traces system and kindly provided by the National Food Chain Safety Office, Hungary.



Supplementary Figure S2 | *Genetic relatedness of livestock-associated methicillin-resistant* Staphylococcus aureus *strains isolated in Hungary.* The UPGMA tree was generated using the cgMLST distance matrix of whole genome sequence data of 120 isolates comprising all horse-related, swine farm originated and human clinical *MRSA CC398* strains. The annotations showing *spa* type, SCCmec type and sample origin are colour coded as follows. *spa* type: , t011; , t034; , other *spa* type. SCC*mec* type: , IVa(2B); , Vc(5C2&5). Origin of sample: , horse-related; , swine environment; , swine professional; , human clinical sample. The identified equine MRSA clusters are also highlighted: , lineage 1 (L1); , lineage 2A, and , lineage 2B (L2a and L2b). The branch with allelic distance greater than 200 was trimmed and indicated accordingly.



Supplementary Figure S3 | *Per-month distribution of Staphylococcus sp. (Staph. sp.), methicillin-sensitive* Staphylococcus aureus *(MSSA) and methicillin-resistant* Staphylococcus aureus *(MRSA) positive cases in the Department and Clinic of Equine Medicine.* The isolates of cases indicated with blank columns were not conserved and hence could not be further investigated.

Supplementary Table S1 | *Quality criteria of whole genome sequence data intended for multilocus sequence typing in BioNumerics software*

Raw data statistics	
Average read quality	> 30.0
Expected coverage	> 30
Q30 frequency	> 60.0
Q30 frequency 1st end	> 70.0
Q30 frequency 2nd end	> 50.0
Raw data statistics after trimming	
Average read quality	> 30.0
Expected coverage	> 30
Q30 frequency	> 60.0
Q30 frequency 1st end	> 70.0
Q30 frequency 2nd end	> 50.0
De novo assembly	
N50	> 27800
Contigs	< 170
Average coverage	> 30.0
Sequence lenght	2.1 - 3.5 Mb
Bases ACGT	> 2561527
Bases N	< 28461
Bases non ACGTN	< 1423
Assembly-free calls	
Average coverage	> 15.0
Multiple alleles	< 133
Perfect matches	> 2256
Present alleles	1954-3246
Assembly-based calls	
Multiple alleles	< 14
Perfect matches	> 2452
Alleles to submit	< 191
Submitted alleles	< 1362
Present alleles	1954-3246
Average locus coverage	> 15
Summary calls	
Unknown alleles	< 39
Multiple alleles	< 26
Discrepant alleles	< 13
Confirmed alleles	> 2196
Present alleles	1954-3246
% core present	> 80.0

Supplementary Table S2 | *Methicillin-resistant* Staphylococcus aureus *clonal complex (CC) 398 strains of pig farm environment, swine professional and human clinical specimen origin selected for whole genome sequencing*

No.	Isolate	Origin	Place of Isolation	Sample Source	Sequence Type	*spa* Type	SCCmec	Virulence Genes	Accession Number
1	F01_E1	FARM 01	Szarvas	environment	398	t011	Vc(5C2&5)		PRJNA901421
2	F02_E1	FARM 02	Bácsalmás	environment	398	t034	Vc(5C2&5)		PRJNA901421
3	F03_E1	FARM 03	Bácsalmás	environment	398	t034	Vc(5C2&5)		PRJNA901421
4	F04_E1	FARM 04	Tedej	environment	398	t4208	Vc(5C2&5)		PRJNA901421
5	F04_H1	FARM 04	Tedej	human nasal swab	398	t011	Vc(5C2&5)		PRJNA901421
6	F05_E1	FARM 05	Ormándlak	environment	398	t034	Vc(5C2&5)		PRJNA901421
7	F06_E1	FARM 06	Dávod	environment	398	t034	Vc(5C2&5)		PRJNA901421
8	F07_E1	FARM 07	Hajdúszovát	environment	398	t011	Vc(5C2&5)		PRJNA901421
9	F08_E1	FARM 08	Törökdomb	environment	398	t011	Vc(5C2&5)		PRJNA901421
10	F08_E2	FARM 08	Törökdomb	environment	398	t034	Vc(5C2&5)		PRJNA901421
11	F08_H1	FARM 08	Törökdomb	human nasal swab	398	t011	Vc(5C2&5)		PRJNA901421
12	F09_E1	FARM 09	Mohács	environment	398	t034	Vc(5C2&5)		PRJNA901421
13	F10_E1	FARM 10	Mezőtárkány	environment	398	t011	Vc(5C2&5)		PRJNA901421
14	F11_E1	FARM 11	Sajószöged	environment	398	t011	Vc(5C2&5)		PRJNA901421
15	F11_H1	FARM 11	Sajószöged	human nasal swab	398	t034	Vc(5C2&5)		PRJNA901421
16	F12_E1	FARM 12	Somogytarnóca	environment	398	t1197	Vc(5C2&5)		PRJNA901421
17	F12_H1	FARM 12	Somogytarnóca	human nasal swab	398	t1197	Vc(5C2&5)		PRJNA901421
18	F13_E1	FARM 13	Hajdúszoboszló	environment	398	t011	Vc(5C2&5)		PRJNA901421
19	F14_E1	FARM 14	Szilfás	environment	398	t034	Vc(5C2&5)		PRJNA901421
20	F15_H1	FARM 15	Pakod	human nasal swab	398	t011	Vc(5C2&5)		PRJNA901421
21	F16_E1	FARM 16	Püspökladány	environment	398	t4208	Vc(5C2&5)		PRJNA901421
22	F17_E1	FARM 17	Kéleshalom	environment	398	t011	Vc(5C2&5)		PRJNA901421
23	F17_H1	FARM 17	Kéleshalom	human nasal swab	398	t011	Vc(5C2&5)		PRJNA901421
24	F18_E1	FARM 18	Kaba	environment	398	t011	Vc(5C2&5)		PRJNA901421
25	F19_E1	FARM 19	Karcag	environment	6268	t034	Vc(5C2&5)		PRJNA901421
26	F20_E1	FARM 20	Derecske	environment	398	t011	Vc(5C2&5)		PRJNA901421

No.	Isolate	Origin	Place of Isolation	Sample Source	Sequence Type	*spa* Type	SCCmec	Virulence Genes	Accession Number
27	F20_E2	FARM 20	Derecske	environment	398	t034	IVa(2B):1		PRJNA901421
28	F21_H1	FARM 21	Murony	human nasal swab	398	t1255	Vc(5C2&5)		PRJNA901421
29	F23_H1	FARM 23	Bicsérd	human nasal swab	398	t034	Vc(5C2&5)		PRJNA901421
30	F24_E1	FARM 24	Csemő	environment	541	t1250	Vc(5C2&5)		PRJNA901421
31	F25_H1	FARM 25	Kistőke	human nasal swab	398	t034	Vc(5C2&5)		PRJNA901421
32	F27_E1	FARM 27	Zomba	environment	398	t011	Vc(5C2&5)		PRJNA901421
33	F27_H1	FARM 27	Zomba	human nasal swab	398	t034	Vc(5C2&5)		PRJNA901421
34	F28_E1	FARM 28	Hódmezővásárhely	environment	398	t011	Vc(5C2&5)		PRJNA901421
35	F28_E2	FARM 28	Hódmezővásárhely	environment	398	t011	Vc(5C2&5)		PRJNA901421
36	F28_H1	FARM 28	Hódmezővásárhely	human nasal swab	398	t034	Vc(5C2&5)		PRJNA901421
37	F28_H2	FARM 28	Hódmezővásárhely	human nasal swab	398	t011	Vc(5C2&5)		PRJNA901421
38	F29_E1	FARM 29	Cibakháza	environment	398	t011	Vc(5C2&5)		PRJNA901421
39	F29_E2	FARM 29	Cibakháza	environment	398	t011	Vc(5C2&5)		PRJNA901421
40	F29_H1	FARM 29	Cibakháza	human nasal swab	398	t011	Vc(5C2&5)		PRJNA901421
41	F30_E1	FARM 30	Lajoskomárom	environment	398	t011	Vc(5C2&5)		PRJNA901421
42	F30_H1	FARM 30	Lajoskomárom	human nasal swab	398	t571	Vc(5C2&5)		PRJNA901421
43	F31_E1	FARM 31	Sárbogárd	environment	398	t034	Vc(5C2&5)		PRJNA901421
44	F32_E1	FARM 32	Dunaszekcső	environment	398	t011	Vc(5C2&5)		PRJNA901421
45	F32_H1	FARM 32	Dunaszekcső	human nasal swab	398	t1451	Vc(5C2&5)		PRJNA901421
46	F33_H1	FARM 33	Katymár	human nasal swab	398	t4571	Vc(5C2&5)		PRJNA901421
47	F34_E1	FARM 34	Ács	environment	398	t034	Vc(5C2&5)		PRJNA901421
48	F34_H1	FARM 34	Ács	human nasal swab	398	t034	Vc(5C2&5)		PRJNA901421
49	F35_E2	FARM 35	Mocsa	environment	398	t034	Vc(5C2&5)		PRJNA901421
50	F36_E1	FARM 36	Borota	environment	398	t011	Vc(5C2&5)		PRJNA901421
51	F36_H1	FARM 36	Borota	human nasal swab	398	t011	Vc(5C2&5)		PRJNA901421
52	F37_E1	FARM 37	Szákszend	environment	398	t4208	Vc(5C2&5)		PRJNA901421
53	F37_H1	FARM 37	Szákszend	human nasal swab	398	t011	Vc(5C2&5)		PRJNA901421
54	F38_E1	FARM 38	Döbrököz	environment	541	t034	Vc(5C2&5)		PRJNA901421
55	F39_E1	FARM 39	Gyulaj	environment	541	t034	Vc(5C2&5)		PRJNA901421
56	F40_E1	FARM 40	Sárbogárd	environment	398	t034	Vc(5C2&5)		PRJNA901421

No.	Isolate	Origin	Place of Isolation	Sample Source	Sequence Type	*spa* Type	SCCmec	Virulence Genes	Accession Number
57	N19018	N/A	Dombegyház	wound	8001	t034	Vc(5C2&5)		PRJNA893357
58	N19030	N/A	Berettyóújfalu	blood culture	398	t4208	Vc(5C2&5)		PRJNA893357
59	N19064	N/A	Körösnagyharsány	wound	398	t011	Vc(5C2&5)		PRJNA893357
60	N19133	N/A	Kondoros	abscess	398	t011	Vc(5C2&5)		PRJNA893357
61	N19145	N/A	Nyírbátor	abscess	1232	t034	Vc(5C2)	*scn, lukS-PV/lukF-PV*	PRJNA893357
62	N19211	N/A	Mohács	wound	398	t034	Vc(5C2&5)		PRJNA893357
63	N19218	N/A	Dombegyház	wound	398	t034	Vc(5C2&5)		PRJNA893357
64	N19280	N/A	Békéscsaba	ear (swab)	398	t011	Vc(5C2&5)	*scn*	PRJNA893357
65	N19281	N/A	Békés	sputum	398	t034	Vc(5C2&5)	*scn*	PRJNA893357
66	N19284	N/A	Tököl	abscess	1232	t034	Vc(5C2)	*scn, lukS-PV/lukF-PV*	PRJNA893357
67	N19294	N/A	Hajdúböszörmény	blood culture	398	t034	Vc(5C2&5)		PRJNA893357
68	N19329	N/A	Nyírtelek	ear (swab)	398	t3275	Vc(5C2&5)		PRJNA893357
69	N19330	N/A	Tiszavasvári	blood culture	398	t4208	Vc(5C2&5)		PRJNA893357
70	N20002	N/A	Kengyel	lower respiratory tract specimen	398	t034	Vc(5C2&5)		PRJNA893357

Supplementary Table S3 | *Equine hospital associated methicillin-resistant* Staphylococcus aureus *clonal complex (CC) 398 strains selected for whole genome sequencing*

No.	Sample ID	Host	Sample Origin	Year of Sampling	Location / Country	Classical MLST Type	*Spa* Type	SCCmec Type	Reference	ENA or SRA Accession
Hygienical Screen of the Department and Clinic of Equine Medicine										
1	E00021807	horse	nasal swab (out)	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
2	E04821708	horse	nasal swab (out)	2017	Clinic 1 (screen)	ST398	t011	IV	this study	-
3	E03761803	horse	nasal swab (out)	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
4	E04541812	horse	nasal swab (in)	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
5	E06541808	horse	nasal swab (in)	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
6	E07151813	horse	nasal swab (in)	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
7	E07151827	horse	nasal swab (in)	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
8	E07551811	horse	nasal swab (in)	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
9	E07551817	horse	nasal swab (in)	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
10	E07551830	horse	nasal swab (in)	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
11	E00331801	-	environment	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
12	E00331803	-	environment	2018	Clinic 1 (screen)	ST398	t011	IV	this study	-
Targeted Sampling of an Equine Hospital-Associated Outbreak										
13	E03111822	horse	nasal swab	2018	Clinic 2	ST398	t011	IV	this study	-
14	E03111833	horse	wound	2018	Clinic 2	ST398	t011	IV	this study	-
15	E02871800	horse	lung	2018	Farm S1	ST398	t011	IV	this study	-
16	E03231801	horse	nasal swab	2018	Farm S2	ST398	t011	IV	this study	-
Targeted Sampling of an Isolated Outbreak										
17	E01001902	horse	nasal swab	2019	Farm S4	ST398	t011	IV	this study	-
18	E01151903	horse	nasal swab	2019	Farm S4	ST398	t011	IV	this study	-
19	E03061901	horse	nasal swab	2019	Farm S4	ST398	t011	IV	this study	-
Isolates of Private Veterinary Practices										
20	E03891801	horse	joint	2008	Clinic 2	ST398	t011	IV	this study	-
21	E02721801	horse	joint	2010	Clinic 2	ST398	t011	IV	this study	-
22	E02721816	horse	wound	2013	Clinic 2	ST398	t011	IV	this study	-
23	E02721819	horse	wound	2013	Clinic 2	ST398	t011	IV	this study	-

No.	Sample ID	Host	Sample Origin	Year of Sampling	Location / Country	Classical MLST Type	*Spa* Type	SCCmec Type	Reference	ENA or SRA Accession
24	E02721802	horse	skin scraping	2010	Farm A	ST398	t011	IV	this study	-
25	E02721803	horse	uterus	2010	Farm B	ST398	t011	IV	this study	-
26	E02720807	horse	wound	2011	Farm C	ST398	t011	IV	this study	-
27	E02721805	horse	wound	2011	Farm D	ST398	t011	IV	this study	-
28	E02721809	horse	wound	2012	Farm E	ST398	t011	IV	this study	-
29	E02721817	horse	wound	2013	Farm F	ST398	t011	IV	this study	-
30	E03891804	horse	wound	2016	Farm G	ST398	t011	IV	this study	-
31	E03891805	horse	skin scraping	2017	Farm H	ST398	t011	IV	this study	-
32	E03891806	horse	skin scraping	2017	Farm I	ST398	t011	IV	this study	-
33	E02791802	horse	wound	2018	Farm J	ST398	t011	IV	this study	-
34	E03891807	horse	wound	2018	Farm J	ST398	t011	IV	this study	-
Isolates of the Diagnostic Laboratory and the Department and Clinic of Equine Medicine										
35	E02980900	horse	fistula	2009	Clinic 1	ST398	t011	IV	this study	-
36	E02721804	horse	wound	2010	Clinic 1	ST398	t011	IV	this study	-
37	E05721000	horse	wound	2010	Clinic 1	ST5857	t011	IV	this study	-
38	E04431100	horse	abdominal exsudate	2011	Clinic 1	ST398	t011	IV	this study	-
39	E05751100	horse	fistula	2011	Clinic 1	ST398	t011	IV	this study	-
40	E01021200	horse	pododermatitis	2012	Clinic 1	ST398	t011	IV	this study	-
41	E06921200	horse	tracheal lavage	2012	Clinic 1	ST398	t011	IV	this study	-
42	E07241300	horse	conjuntiva	2013	Clinic 1	ST398	t011	IV	this study	-
43	E08441300	horse	wound	2013	Clinic 1	ST398	t011	IV	this study	-
44	E03301400	horse	abscess	2014	Clinic 1	ST398	t011	IV	this study	-
45	E04721400	horse	wound	2014	Clinic 1	ST398	t011	IV	this study	-
46	E00571500	horse	wound	2015	Clinic 1	ST398	t011	IV	this study	-
47	E02611500	horse	wound	2015	Clinic 1	ST398	t011	IV	this study	-
48	E00991600	horse	lung	2016	Clinic 1	ST398	t011	IV	this study	-
49	E04351600	horse	wound	2016	Clinic 1	ST398	t011	IV	this study	-
50	E01271700	horse	thoracic exsudate	2017	Clinic 1	ST398	t011	IV	this study	-

No.	Sample ID	Host	Sample Origin	Year of Sampling	Location / Country	Classical MLST Type	*Spa* Type	SCCmec Type	Reference	ENA or SRA Accession
European Equine-Related CC398-SCC*mec*IV MRSA Whole Genome Sequence Data										
51	3029/2013	horse	sinus	2013	Austria	ST398	t011	IV	Loncaric et al., 2019	-
52	3260/2013	horse	fistula	2013	Austria	ST398	t011	IV	Loncaric et al., 2019	-
53	1735/2014	horse	wound	2014	Austria	ST398	t011	IV	Loncaric et al., 2019	-
54	1874/2014	horse	wound	2014	Austria	ST398	t011	IV	Loncaric et al., 2019	-
55	2051/2014	horse	wound	2014	Austria	ST398	t011	IV	Loncaric et al., 2019	-
56	3779/2014	horse	wound	2014	Austria	ST398	t011	IV	Loncaric et al., 2019	-
57	3855/2014	horse	wound	2014	Austria	ST398	t011	IV	Loncaric et al., 2019	-
58	4042/2014	horse	wound	2014	Austria	ST398	t011	IV	Loncaric et al., 2019	-
59	1226/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
60	1758/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
61	2200/2015	horse	sinus	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
62	2202/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
63	2297/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
64	2541/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
65	2834/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
66	3071/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
67	3290/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
68	3586/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
69	3587/2015	horse	abscess	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
70	3861/2015	horse	fistula	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
71	3877/2015	horse	wound	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
72	3915/2015	horse	nasal swab	2015	Austria	ST398	t011	IV	Loncaric et al., 2019	-
73	0059/2016	horse	mouth	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
74	0396/2016	horse	wound	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
75	0407/2016	horse	wound	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
76	0561/2016	horse	wound	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
77	0588/2016	horse	periorbital wound	2016	Austria	ST398	t1985	IV	Loncaric et al., 2019	-

No.	Sample ID	Host	Sample Origin	Year of Sampling	Location / Country	Classical MLST Type	*Spa* Type	SCCmec Type	Reference	ENA or SRA Accession
78	0625/2016	horse	wound	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
79	0760/2016	horse	fistula	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
80	1005/2016	horse	tracheal lavage	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
81	1225/2016	horse	wound	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
82	1281/2016	horse	nasal swab	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
83	1287/2016	horse	wound	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
84	1675/2016	horse	wound	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
85	1975/2016	horse	wound	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
86	2182/2016	horse	fistula	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
87	2201/2016	horse	wound	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
88	2700/2016	horse	uterus	2016	Austria	ST398	t011	IV	Loncaric et al., 2019	-
89	0010/2017	horse	nasal swab	2017	Austria	ST398	t011	IV	Loncaric et al., 2019	-
90	1418/2017	horse	tracheal lavage	2017	Austria	ST398	t011	IV	Loncaric et al., 2019	-
91	2517/2015	horse	wound	2017	Austria	ST6654	t011	IV	Loncaric et al., 2019	-
92	2646/2017	horse	fibrin	2017	Austria	ST398	t011	IV	Loncaric et al., 2019	-
93	3214/2017	horse	wound	2017	Austria	ST398	t011	IV	Loncaric et al., 2019	-
94	1840/18	horse	vein content	2018	Austria	ST398	t011	IV	Loncaric et al., 2019	-
95	2933/18	horse	wound	2018	Austria	ST398	t011	IV	Loncaric et al., 2019	-
96	3058/18	horse	wound	2018	Austria	ST398	t6867	IV	Loncaric et al., 2019	-
97	ID-99	horse	n/a	2008	Belgium	ST398	t011	IV	Sharma et al., 2016	ERR1437623
98	Case_E	human	wound	2014	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823544
99	Case_I	human	wound	2014	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823548
100	Case_D	human	wound	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823543
101	H002	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823551
102	H042	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823552
103	H043	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823553
104	H044	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823554
105	H046	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823555
106	H155	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823556

No.	Sample ID	Host	Sample Origin	Year of Sampling	Location / Country	Classical MLST Type	*Spa* Type	SCCmec Type	Reference	ENA or SRA Accession
107	H157	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823557
108	H160	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823558
109	H161	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823559
110	H163	horse	nasal swab	2015	Denmark	ST398	t011	IV	Islam et al., 2017	ERR1823560
111	IMT33368	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUTZ01000000
112	IMT33391	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUTY01000000
113	IMT33826	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUUA01000000
114	IMT33828	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUUB01000000
115	IMT33861	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUUC01000000
116	IMT33862	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUUD01000000
117	IMT33997	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUUE01000000
118	IMT34080	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUXH01000000
119	IMT34209	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUXI01000000
120	IMT34426	horse	nasal swab	2014	Germany	ST398	t011	IV	Walther et al., 2018	PUXJ01000000
121	IMT38888	horse	nasal swab	2014	Germany	ST398	t011	IV	PATRIC Database	QBFM01000000
122	IMT36995	horse	nasal swab	2015	Germany	ST398	t011	IV	Walther et al., 2018	PUXK01000000
123	IMT37082	horse	nasal swab	2015	Germany	ST398	t011	IV	Walther et al., 2018	PUXL01000000
124	IMT37264	horse	nasal swab	2015	Germany	ST398	t011	IV	Walther et al., 2018	PUXM01000000
125	IMT37277	horse	nasal swab	2015	Germany	ST398	t011	IV	Walther et al., 2018	PUXN01000000
126	IMT37325	horse	nasal swab	2015	Germany	ST398	t011	IV	Walther et al., 2018	PUXO01000000
127	IMT37340	horse	nasal swab	2015	Germany	ST398	t011	IV	Walther et al., 2018	PUXP01000000
128	IMT37426	horse	nasal swab	2015	Germany	ST398	t011	IV	Walther et al., 2018	PYBG01000000
129	IMT37510	horse	nasal swab	2015	Germany	ST398	t011	IV	Walther et al., 2018	PUXQ01000000
130	IMT38358	horse	conjuntiva	2016	Germany	ST398	t011	IV	Soimala et al., 2018	QITK01000000
131	IMT38422	horse	conjuntiva	2016	Germany	ST398	t011	IV	Soimala et al., 2018	QITL01000000
132	IMT38431	horse	conjuntiva	2016	Germany	ST398	t011	IV	Soimala et al., 2018	QITM01000000
133	IMT38587	horse	conjuntiva	2016	Germany	ST398	t011	IV	Soimala et al., 2018	QITN01000000
134	IMT38594	horse	conjuntiva	2016	Germany	ST398	t011	IV	Soimala et al., 2018	QITO01000000
135	IMT38653	horse	conjuntiva	2016	Germany	ST398	t011	IV	Soimala et al., 2018	QITP01000000

No.	Sample ID	Host	Sample Origin	Year of Sampling	Location / Country	Classical MLST Type	*Spa* Type	SCCmec Type	Reference	ENA or SRA Accession
136	C3865	horse	bodily fluid	2005	*Spa*in	ST398	t011	IV	PATRIC Database	SRR1300904
137	C4668	horse	bodily fluid	2011	*Spa*in	ST398	t011	IV	PATRIC Database	SRR1218555
138	17Gst018	human	nasal swab	2017	Switzerland	ST398	t011	IV	Kittl et al., 2020	SRR9734211
139	17Gst094_nose	human	nasal swab	2017	Switzerland	ST398	t011	IV	Kittl et al., 2020	SRR9734217
140	17Gst112	human	nasal swab	2017	Switzerland	ST398	t011	IV	Kittl et al., 2020	SRR9734218
141	17Gst165	human	nasal swab	2017	Switzerland	ST398	t011	IV	Kittl et al., 2020	SRR9734215
142	17Gst166	human	nasal swab	2017	Switzerland	ST398	t011	IV	Kittl et al., 2020	SRR9734216
143	17KM0012	horse	lung	2017	Switzerland	ST398	t011	IV	Kittl et al., 2020	SRR9734202
144	17KM0743	horse	wound	2017	Switzerland	ST398	t011	IV	Kittl et al., 2020	SRR9734201
145	17KM2889	horse	nasal swab	2017	Switzerland	ST398	t011	IV	Kittl et al., 2020	SRR9734204
146	GD2010-169	horse	nasal swab	2010	The Netherlands	ST398	t011	IV	PATRIC Database	SRR1302881
147	PHE01	horse	n/a	2008	United Kingdom	ST398	t011	IV	Sharma et al., 2016	ERR1450615
148	PHE02	horse	n/a	2008	United Kingdom	ST398	t011	IV	Sharma et al., 2016	ERR1450616
149	PHE03	horse	n/a	2008	United Kingdom	ST398	t011	IV	Sharma et al., 2016	ERR1450617

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