University of Veterinary Medicine Budapest
Doctoral School of Veterinary Sciences

Molecular characterisation of Staphylococcus aureus strains isolated from rabbits

PhD thesis

Zoltán Német DVM

2020

Supervisor and consultants:

Imre Biksi, DVM, habil. PhD

University of Veterinary Medicine Budapest

Department of Pathology

Supervisor

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Zoltán Német

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Abbreviations

AB – assembly-based

AF – assembly free

aHV - atypical highly virulent

CC8 – clonal complex 8

CFU – colony-forming unit

CUT – cutaneous lesion

ENA – European Nucleotide Archives

HV – highly virulent

INT – intestinal lesion

LV – low virulence

MAST – mastitis

MET – metritis

MLST - multilocus sequence typing

MLST – multilocus sequence typing

MRSA - methicillin-resistant *Staphylococcus aureus*

NGS – next generation sequencing

PATRIC – Pathosystems Resource Integration Center

PCR – polymerase chain reaction

PRSA - penicillin-resistant *Staphylococcus aureus*

RAST – Rapid Annotation using Subsystem Tecnology

RESP – respiratory

SEPT – septicaemia

spa – staphylococcal protein A

wgMLST – whole-genome multilocus sequence typing

WGS – whole-genome shotgun

1. Summary

Staphylococcosis is one of the most important diseases in commercial rabbit meat production. *Staphylococcus aureus* is a versatile, opportunistic pathogenic microorganism, which is able to persist and multiply in the animals, humans and environment of farms, and causes a wide spectrum of diseases in both humans and animals.

Scientific studies investigating rabbit staphylococcosis revealed great versatility in both clinical, pathological and microbiological aspects of the disease. International dissemination of virulent variants of the pathogen had been reported, and the only feasible solution for outbreaks caused by such agents was complete depopulation of contaminated units. Present study was originated from the request of Hungarian rabbit breeders and meat producers, because production losses, and ineffective treatment programmes clearly demonstrated, that veterinary practice needed substantial improvement in this regard.

The aim of this study was to gain information on the clinical situation on rabbit farms, collect isolates, and characterise them in order to facilitate more effective treatment and preventive measures in commercial production. Hundreds of farm visits and thousands of necropsies resulted in an isolate archive containing more than 500 isolates in 10 years. A molecular genotyping method, a multiplex PCR system was introduced as part of the standard diagnostic protocol. The highly virulent variant was detected on some Hungarian farms, these strains later proved to be very similar to other ST121/t645 strains isolated in Italian and Spanish rabbit farms. The most abundant genotype on Hungarian farms is an atypical highly virulent variant, similar genotype was only detected in one outbreak in Belgium, in 1996. The organotropism of *Staphylococcus* strains was compared to the genotype of the isolate, significant differences were revealed between virulence types. Highly virulent variants are strongly correlated to septicaemia. Low virulence and non-aureus *Staphylococcus* strains are much more likely to only cause local lesions on the skin.

The next step was to use whole-genome sequencing for a more detailed comparison between virulent variants. We compared the two draft genomes in two different approaches. Pairwise alignment of the two drafts revealed that the two virulent variants are in fact two very different, far related variants of *Staphylococcus aureus*. Comparing 63 virulence-related genes we concluded that extracellular enzymes of the two genotypes are very similar, but the whole enterotoxin gene cluster – which is the basis of differentiation between high and low virulence strains in the multiplex PCR – is completely absent from the atypical highly virulent strain. Surface components and adhesion factors

were proven to be notably different between highly virulent and atypical highly virulent strains.

The final step was the whole-genome sequencing of 50 Stapylococcus isolates, assembly of draft genome sequences, publicating all data to open digital repositories. The whole-genome multilocus sequence typing of 64 rabbit-originated Staphylococcus isolates representing 4 European countries with whole-genome MLST reinforced the clonal origin and international dissemination of highly virulent ST 121 strains, demonstration of two new clonal clustrers (aHV-ST5993, LV-ST2855) of the pathogen.

Identification of rabbit farms contaminated with highly virulent variants contributed to the decision of depopulating these commercial units, after disinfection and repopulation the ST121/t645 highly virulent variants were never detected in Hungary. The whole-genome sequencing provided useful epidemiologic information, and a large amount of sequence data for the practice and science of rabbit medicine.

Összefoglaló

A staphylococcosis a modern nagyüzemi nyúlhús termelésben az egyik legfontosabb fertőző betegség. A *Staphylococcus aureus* egy változatos opportunista patogén mikroorganizmus, amely az üzemi termelő állományokban, környezetükben és a dolgozókban egyaránt képes tartós túlélésre és szaporodásra. A kórokozó számos kórkép kialakításában játszhat szerepet állatokban és emberekben egyaránt.

A házinyúl staphylococcosisát vizsgáló tudományos munkák klinikai, kórbonctani és járványtani szempontból is változatos képet mutatnak. A nagy virulenciájú változatok jelenlétét minden jelentős nyúltermelő országban igazolták, az ilyen kórokozók által kirobbantott járványok elfojtására az érintett állományok teljes felszámolása volt az egyetlen megoldás. A jelen dolgozatban összefoglalt munka a magyar nyúltenyésztők kérésére indult. A termelésben elszenvedett veszteségek és a kezelési programok sikertelensége miatt az a felkérés fogalmazódott meg, hogy az állatorvosi gyakorlat ezen ágában történjenek jelentős fejlesztések.

A munka célja az volt, hogy az állományok felmérése, a baktériumtörzsek összegyűjtése és vizsgálata olyan információkkal szolgáljon, amelyekre hatékony kezelési és megelőzési módszereket lehet alapozni. Tíz év alatt több száz teleplátogatás és több ezer kórbonctani-diagnosztikai vizsgálat eredményeként létrejött egy jelenleg több mint 500 izolátumot tartalmazó törzsgyűjtemény. Molekuláris genotipizálásra egy multiplex PCR rendszert vezettünk be a standard diagnosztikai protokollba. A nagy virulenciájú változatot azonosítottuk néhány magyar állományban, később ezek a törzsek az olasz és spanyol telepeken talált ST121/t645 törzsekkel nagyfokú genetikai hasonlóságot mutattak. A hazai telepeken a leggyakoribb genotípus egy atipikus nagy virulenciájú vátozat, hasonló törzseket Belgiumban izoláltak egy járvány során 1996-ban. A különböző szervrendszerekből kimutatott különböző virulencia változatok adatainak statisztikai elemzése szignifikáns összefüggéseket fedett fel. A nagy virulenciájú változatok erősen korreláltak a septicaemiával. Az alacsony virulenciájú változatok és a nem *Staphylococcus aureus* fajba tartozó *Staphylococcus* törzsek jellemzően helyi bőrelváltozásokkal vannak kapcsolatban.

Következő lépésként teljes genom szekvenálással végeztünk részletes összehasonlítást a virulens változatok között. Egy tipikus és egy atipikus nagy virulenciájú törzs genomját hasonlítottuk össze két módszerrel. A teljes genomszekvenciák párhuzamos illesztése azt mutatta, hogy a két változat egymástól jelentősen különbözik, a két genotípus a *Staphylococcus aureus* faj két egymástól távoli változatát képviseli. 63

virulenciagén nukleotid bázissorrendjének összehasonlításával azt állapítottuk meg, hogy az extracelluláris enzimeket kódoló gének általában nagyon hasonlóak, de az enterotoxin gén klaszter – amely a nagy és kis virulenciájú változatok elkülönítésének alapja a multiplex PCR rendszerben – teljesen hiányzik az atipikus nagy virulenciájú törzsből. A sejtfelületi fehérjék és adhéziós faktorok tekintetében jelentős különbözőséget találtunk a két genotípus között.

Utolsó lépésként 50 újabb törzs teljes genom szekvenálását, illesztését és nyílt génbankban való közzétételét végeztük el. A saját törzsek mellett a génbankokban elérhető összes házinyúl eredetű, négy európai országból származó *Staphylococcus aureus* izolátum, összesen 64 teljes genomszekvenciát hasonlítottunk össze teljes genom MLST módszerrel. A vizsgálat megerősítette a virulens ST121 változat klonális eredetét, emellet két eddig ismeretlen klonális klasztert (aHV-ST5993, LV-ST2855) azonosítottunk.

A virulens kórokozó változattal terhelt telepek azonosítása hozzájárult az érintett állományok felszámolását eredményező döntésekhez, fertőtlenítésük és újratelepítésük után az ST121/t645 kórokozó genotípus többet nem volt izolálható vizsgálataink során. A teljes genom szekvenálás hasznos járványtani információkkal, és jelentős mennyiségű szekvencia adattal járult hozzá a gyakorlati és tudományos házinyúl egészségügy területéhez.

2. Introduction

2.1. The importance of the rabbit

The European rabbit (*Oryctolagus cuniculus*, Leporidae family, Lagomorph order) has been used in European agriculture for at least 3000 years. The evolution of husbandry passed numerous stations during the centuries, and resulted in a highly efficient farming system, comparable to technologies developed for other producing species (Lebas, 1997). The rabbit is a major source of food and fur, and an indispensable asset in life sciences and pharmaceutical industry (McNitt et al., 2013).

The digestive and reproductive physiology of the species outlines a potent meat-producing animal. Being a highly specialized herbivore, the rabbit is able to utilize plant fibres and low-quality proteins with a high conversion capacity. The high prolificacy and quick growth permits a slaughter to breeder body weight ratio close to avian producing species (Lebas, 1997).

The special qualities of the rabbit meat elevate this product above other mass-produced protein sources, the high protein content, excellent digestibility and optimal amino acid composition makes this meat ideal for human consumption. The fatty-acid composition enables this product even as a functional food (Dalle Zotte and Szendrő, 2011).

The Statistic Division of the FAO indicates a total world production of 1,4 million tonnes per year. Hungary in 2017 produced nearly 5000 tonnes of rabbit meat, almost all of this for export, which is approximately 20% of all exported rabbit meat in Europe (FAOSTAT, 2018).

Despite the long history of farming, advanced techniques and extended research, rabbit farming remained a very insecure business. The acceptable mortality on average farms is 0,5-1 % per week, which would be considered disastrous in poultry or pig industries (Rosell and de la Fuente, 2009). Antibiotic use is very high in comparison, with the current slow pace of decrease, the forthcoming and inevitable drastic restrictive legislation of the EU can possibly drive the whole European rabbit industry to a catastrophic situation (Agnoletti et al., 2018).

The animal and it’s meat are equally outstanding natural resources, but future perspectives and success highly depends on the efforts made in related research and development (Cullere and Dalle Zotte, 2018). This makes veterinary diagnostics and

research very important in both the present and future for the rabbit industry. Staphylococcosis has a major economic impact on rabbit farming. Infections caused by virulent strains result in severe, often fatal diseases (Hermans et al., 2003). Gaining knowledge on the organism seems to be the only possibility to produce an effective and lasting defense strategy against this pathogen.

2.2. The history of the pathogen

Staphylococcus aureus is a Gram-positive, round-shaped bacterium credited to Rosenbach in 1884 – is a definition on facts, although it does not contain the full story of the discovery of this pathogen with an increasingly alarming role nowadays.

The story begins years earlier, when Alexander Ogston, full surgeon to the Aberdeen Royal Infirmary, visited his collegue, 1st Baron Joseph Lister, professor of surgery at the University of Glasgow. Lister had been working to implement the novel ideas of French chemist, Louis Pasteur. The role of microorganisms in food spoilage was proven, even perfect elimination of these invisible creatures had been solved, but these methods were not applicable on living humans. Inspired by this fruitful meeting, with the experience of overseas studies and the plentiful clinical skills possessed by Ogston, he wished to find out the precise reason of traumatic wound infections. He was so devoted for the cause, that he built a laboratory in his backyard shed, and started to investigate the pus of human wound infections. His contemporaries' (Robert Koch, J. C. Ewart, etc.) experiments and publications, and a Zeiss microscope, founded by the £50 grant from the British Medical Association, have greatly contributed to the discovery the pathogen. To him we owned the phrase *Staphylococcus*, as he named the bacteria he observed in the pus. Ogston also managed to grow staphylococci in artificial cultures, and transferred the cultures into animals causing the same disease, which provided the ultimate evidence of its' infectious nature (Newsom, 2008). He found two forms of micrococcus, one he named Streptococcus – caused a more violent inflammation - and *Staphylococcus*.

Ogston had a difficult time convincing the medical professors in the UK, finally he presented his findings in a surgical congress in Berlin. The audience was so impressed, not just on his scientific outcome, but his fluent German, that he was right off accepted as Fellow of the German Surgical Society. This acknowledgement convinced the British scientific community to accept the findings of Ogston.

In 1882 Ogston was entrust with the title of Regius Professor of Surgery in Aberdeen, consequently fulfilling his obligations deprived him from further experiments.

So, it happened, that a German scientist, Friedrich Julius Rosenbach was the first who properly discriminated and characterized the two *Staphylococcus* bacteria, and named them *S. aureus* and S. albus based on the colour of them colonies. Anyway, at the golden age of the discovery for bacteria, Alexander Ogston was one of a major influencer, and the shed in his garden was the most important venue in the bacteriology research of the UK.

2.2.1. Livestock-associated MRSA

Staphylococcus aureus is also of major public health importance. Pathogen-induced diseases are likely to be as old as humanity (Waness, 2010). The first scientific case studies were already born in the 19th century (Lowy, 1998). Antibiotics become more and more widely accessible after the World War II, and the doctors tend to prescribe antibiotics for almost all diseases. This frequent usage of antibiotics obviously brought the inappropriate use, like incomplete treatment, which - in just a few years - leads to antibiotic resistant *Staphylococcus aureus* strains.

The spread of antibiotics has been a breakthrough in treating some diseases, but in the United States, penicillin-resistant *Staphylococcus aureus* (PRSA) strains appeared just four years after the introduction of penicillin in 1943. In 1959, a new antibiotic, methicillin was launched to the market, and two years was enough to methicillin-resistant *Staphylococcus aureus* (MRSA) to emerge. Methicillin was introduced to treat PRSA. In a quick period, MRSA spread all over the world, mainly occuring in hospitals, and where an intense usage of antibiotics is in practice (Waness, 2010). The pathogen has also been detected in livestock and slaughterhouses in Europe, Canada and the Far East (Vandenbroucke-Grauls and Beaujean, 2006; Wulf and Voss, 2008). The presence of MRSA strains has already been demonstrated in a Hungarian cattle farm (Juhász-Kaszanyitzky et al., 2007), and there is no reference to their presence in domestic rabbit stocks in the available literature. The MRSA strains are the so-called can be identified by PCR method for the detection of the mecA gene (Unal et al., 1992). These incidences lead to the problem of livestock-associated MRSA.

Intense awareness needed to livestock-associated MRSA lineages as they can represent a major public health threat. The resistance is based on a mutant version of mecA gene, which encode the penicillin-binding protein PBP2a (Morgan, 2008). The host specificity of the *S. aureus* is a trait that can evolve rapidly and lineage-specific which could be a major epidemic threat. Some studies suggest that the variation of mobile genetic elements plays important role in host specificity. The pathogenicity islands of *S. aureus*

are mobile genetic elements, that carry host-specific variants of virulence genes. The acquisition and loss of various pathogenicity islands contribute to the colonising and infectious ability of *S. aureus* (Peton and Le Loir, 2014). The presence of MRSA is already reported in several companion species such as horse, pig, and chinchilla, and in all main livestock species such as ruminants, swine and poultry.

Sakwinska et al. evidenced a rare humans-to-cows shift in case of a common staphylococcal lineage, the *S. aureus* clonal complex 8 (CC8). CC8 is a frequent host of the staphylococcal cassette chromosome mec genomic island, and such isolates often occur in clinical samples. The authors draws attention that considering the frequent occurrence of mastitis, the danger of the bovine-related CC8 MRSA could be a risk factor of either human and veterinary medicine (Sakwinska et al., 2011). A zoonosis study (Shepheard et al., 2013) with multilocus sequence typing (MLST) method, encompass more than three thousand isolates, and identified 15 likely historical switching events. Results demonstrated that a human-specific lineage can appear from an animal host, as two human-associated clade candidates have been identified with livestock-associated ancestors. These data also emphasise that *S. aureus* has the ability to adapt novel hosts, despite the long isolation period in a single species.

A scientific report of Italian industrial rabbit holding has been described the first occurrence of MRSA ST398 in 2014 (Agnoletti et al., 2014). In a comprehensive research 2500 rabbits in 40 farms were examined, and more than 1000 isolates were collected. The authors evidenced the first time that MRSA ST398. This variant is a pig derived clone, that circulates intensively in livestock and farmers from the Netherlands since 2004. This strain has penetrated rabbit meat production, affecting not just the rabbit, but the farmers and even their relatives. Thus, the importance of the early tracing of MRSA lineages in rabbit meat producing farms become important, and may prevent the further spread of the pathogen.

2.3. The disease

Before the discovery of penicillin (Fleming, 1929) invasive staphylococcosis was often fatal. The extraordinary ability of this microorganism to gain resistance against antibiotics resulted a struggling competition between the bacterium and humans, and science and medicine constantly fails to deliver a permanent solution for this problem (Ji, 2014).

Staphylococcus aureus is one of the most important opportunistic pathogens, capable of infecting humans and a large variety of domesticated animals. *S. aureus* can be found in healthy carriers in animals and in humans, and can induce a wide scale of infections. Almost all warm-blooded animals could carry the pathogen, and can be infected by *S. aureus*. *S. aureus* carriage and infection was also reported in reptiles (Adkesson et al., 2007).

This disease in the domesticated European rabbit (*Oryctolagus cuniculus*) is usually presented as a subacute or chronic, purulent and necrotizing inflammation in different organ systems, manifested in an endemic pattern within the flock. Most commonly the infection of the skin, the subcutaneous tissue, the respiratory system and the mammary gland can be observed in practice. Neonatal septicaemia and mastitis of lactating does also frequently develop (Vetési, 1990). Staphylococcosis is a serious problem in rabbit farming. Kit mortality, premature elimination and replacement of diseased breeders, and slaughterhouse condemnations cause substantial losses to the industry (Hermans et al., 2003). Staphylococcosis can affect rabbits at any age, it infects the skin, the mammary glands or any other organ, ultimately causing suppurative lesions or septicaemia. High mortality in suckling rabbits, increased rate of slaughterhouse condemnations and high culling rate of breeding animals can cause substantial economic losses.

Staphylococcus aureus can be found at any rabbit farm, but the amount of losses, and the success of treatments (antibiotics, vaccination, improved hygienic programs) shows great variation in different farms. Research in the last decades has revealed that low virulence (of human or poultry origin) and high virulence *Staphylococcus aureus* strains can be distinguished. Differentiation can be performed by phage typing or by detections of specific genes with PCR. In practice the latter method is used more frequently. Effective treatment against highly virulent strains has not yet been set up, so the only choice to avoid inevitable losses is to prevent the contamination of the flock, or in case of an ongoing epidemic, to repopulate the herd. Latter may be impossible in case of

a genetic nucleus population. It is therefore necessary to determine whether the pathogen detected belongs to the low or to the high virulence group.

Dermatitis and abscess formation, mastitis, respiratory diseases, urogenital infections and septicaemia can be caused by this organism in a variety of host species. Staphylococcal food poisoning (SFP) in humans is also an emerging problem. Other *Staphylococcus* species are described to be related with similar conditions of different species (Peton and Le Loir, 2014).

For decades, the disease has been present in domestic rabbit sites (Vetési Ferenc, 1990) rabbit breeding countries in Europe (Holliman and Girvan, 1986; Okerman et al., 1984) and in the United States (Hagen, 1963). In France, acute diseases were first observed in 1982, but they were able to keep the level losses at a low level until the early 1990s by basic veterinary treatments. Since 1992, the number of cases has multiplied, probably due to the increasing intensity of production technology, and the emergence of the few modern hybrid breeds. After 2002, the pathogen is resistant against several antibiotic agents, and with the proliferation of high virulence strains, the disease has again became a serious problem (Boucher and Nouaille, 2002).

S. aureus has the ability to infect many animal species due to the more than 30 virulence factors which it can produce. These virulence factors contribute to the establishment and maintenance of the staphylococcosis. The pathogen adheres to the colonisation site with cell surface molecules and provide a reservoir to invade the host tissues. Once *S. aureus* adheres, it can grow and produce several enzymes to penetrate the tissues. Enzymes like proteases, lipases, elastases enable *S. aureus* to destroy and feast on the host. *S. aureus* also produces toxins, among these there are exfoliative ones, leucotoxins and superantigens (Vancraeynest et al., 2006). Superantigen activity is useful to decoy and evade the immune system, and allows the persistence of the pathogen on multiple sites of infection (Gordon and Lowy, 2008). Exfoliative toxins prevent epidermal exfoliation, leucotoxins are associated with soft-tissue infections.

Staphylococcus aureus is a facultative, opportunistic pathogen, therefore presumably predisposing factors are needed for the development of the clinical symptoms defining staphylococcosis. Based on the few available publications and our own practical experience, high humidity in the stable, wet litter, and mechanical injuries predispose to develop sepsis and purulent skin inflammation under 1 week of age. Mechanical injuries may occur due to inadequate litter material, or in case the milking time is not restricted, thus the nursing mother's frequent visits may damage the kits (Adlam and Thorley, 1976; Boucher and Nouaille, 2002; Corpa et al., 2010; Hagen, 1963; Hermans et al., 2000a;

Holliman and Girvan, 1986; Marcato and Rosmini, 1986). These factors are common in large-scale production, even in the most advanced maintenance technology.

The epidemic staphylococcosis can occur both in herds with high or low technological and hygienical standards, so some believe that the spread and damage of the disease depends more on the pathogen-host interaction rather than on the interaction between the pathogen and the environment or the host and environment (Corpa et al., 2010; Hermans et al., 2000a).

The newborn animals with *Staphylococcus aureus* infection develop purulent inflammatoric foci in the subcutaneous connective tissue at the abdomen, specifically in the navel region, in the front limb, and in the lower mandible. These areas are most likely to suffer superficial epithelial damage during birth. The staphylococci colonising the maternal mucosa and skin adhere to the subepithelial proteins, and the healing surface layer endorses the minuscule bacterial colony. From 3 to 5 days of age abscesses are formed from these foci, but these abscesses are already covered by the hair, hence obscured from the superficial observer. Mortality and obvious lesions develop for the end of the second week of life (Figure 1) (Hagen, 1963). Most rabbits develop sepsis due to dermatitis (Okerman et al., 1984).

1. Figure: ***Extensive purulent dermatatis with abscess formation in a 2 weeks old rabbit.***

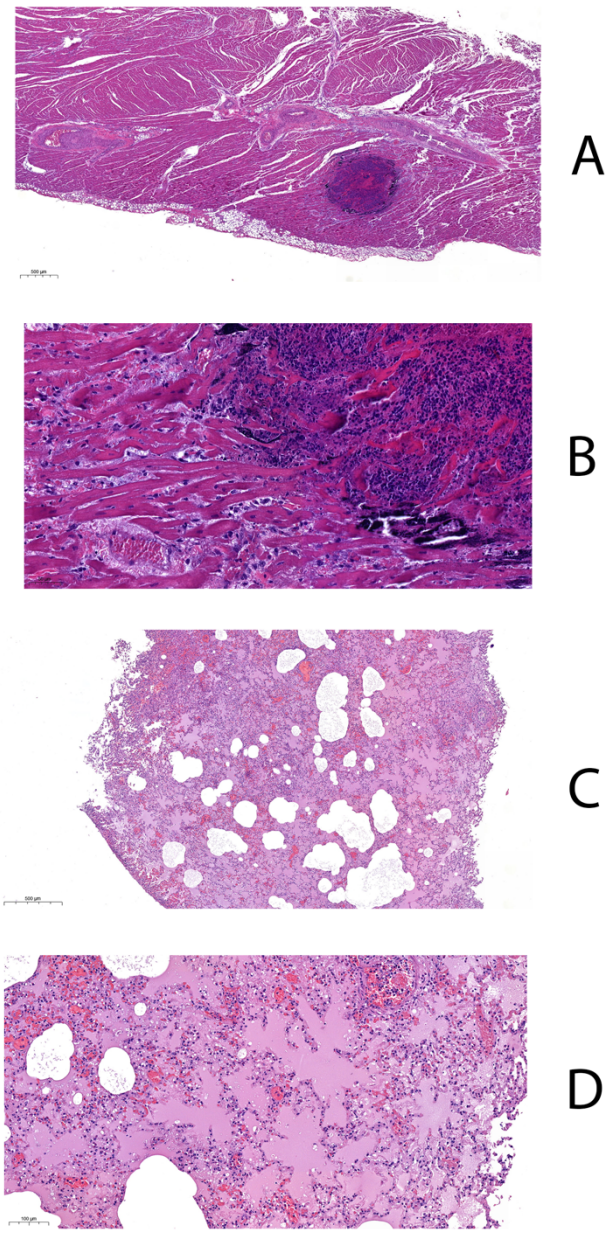
Superficial necrosis in the skin can be proved by histopathological examination. Neutrophil granulocytic infiltration and bacterial forms are also present (Marcato and Rosmini, 1986). In case of sepsis inflammatory-necrosis, or necrotic foci occur in various organs. Myocardial infarction can happen without inflammatory phenomena (Corpa et al., 2010; Hagen, 1963).

The most frequent pathology observed in breeding animals is mastitis (Figure 2). Infection occurs during lactation, ascending infection is usually caused by the bacteria that colonize the epidermis, but abscess caused by haematogenic infection may also arise. Epithelial damage caused to the softened skin of the teats after feeding greatly facilitates colonisation of the area. Some animals get the infection right after the very first parturition, while others show no symptoms after many producing cycles. If the doe becomes affected in the early stages of the production cycle, the offspring usually perishes due to starvation. Healed animals are often re-infected, and in many cases, they do not care for the litter. In addition to death due to infection of the kits, shortages caused by agalactia also cause significant losses (Adlam and Thorley, 1976; Vetési Ferenc, 1990; Corpa et al., 2010).

2. Figure: ***Purulent mastitis in a rabbit doe****. (The photo is the author's own property.)*

Mastitis caused by *Staphylococcus aureus* can be clinically acute or chronic. The acute form of mastitis causes warm and flushed mammary glands, which becomes cyanotic, a necrotic-purulent inflammation can be observed in the glands. The acute form spreads rapidly in the herd, and the affected litters die of starvation (Adlam and Thorley, 1976; Holliman and Girvan, 1986). Occasionally, the sick mother dies within hours, but the disease may be developed into a chronic form (Corpa et al., 2010; Zumpt, 1976). In chronic case, the mammary gland is permeated by the connective tissue, and within 2-3 weeks it develops abscesses of 2 to 10 cm in diameter. The pus can be excreted to through the aroded skin or through a fistule (Adlam and Thorley, 1976). In some cases, the superficial

skin inflammation around the nipple can also be observed (Corpa et al., 2010; Okerman et al., 1984).



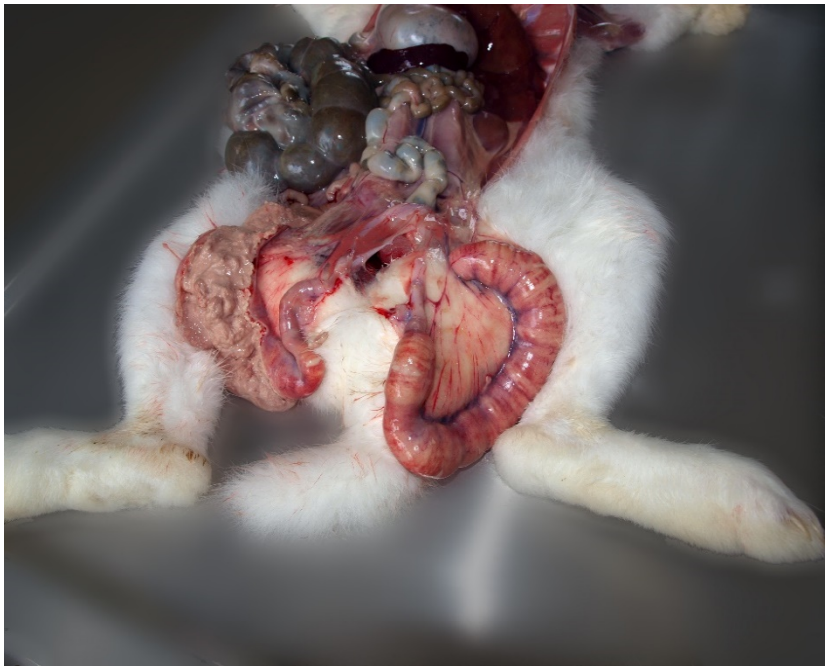
3. Figure 4. Figure ***Focal myocarditis (A: 100x, B: 400x) and pneumonia (C: 100x, D: 400x) in rabbit caused by Staphylococcus aureus. Hematoxilin-eosin.***
(The photos are the author's own property.)

Another important cause of the premature loss of breeding animals is the development of pododermatitis ulcerosa, also called sore hocks (Figure 3). Mothers and bucks are most often culled because of this form of staphylococcosis (Rosell and de la Fuente, 2009). Some studies had shown that the *Staphylococcus aureus* can be detected in 100% (Okerman et al., 1984) and 95% (Rosell and de la Fuente, 2009) of the characteristic lesions. Inadequate cage and wet litter material increase the incidence of the sore hocks, and the heavy weighted breeds are more susceptible to such disease (Boucher and Nouaille, 2002; Corpa et al., 2010; Devriese et al., 1981; Drescher and Schlender-Böbbis, 2010; Vannuffel et al., 1995). The lesion generally appears under the bulging features of the proximal epiphysis of the metatarsus. Hyperplasia of the epithelium and parakeratosis appears first due to increased pressure, later ischemic tissue necrosis can occur. The proliferation of *S. aureus* at the area of the dead skin transforms the process into necrotic-purulent inflammation. In some cases the necrosis of the soft tissues extends so deep, that the bones of the foot are breaching the surface (Corpa et al., 2010; Marcato and Rosmini, 1986; Vetési, 1990).

5. Figure: ***Different stages of staphylococcal ulcerative pododermatitis on breeding does****.*
(The photo is the author's own property.)

Purulent metritis (Figure 4) and abortion may also occur as a result of *Staphylococcus aureus* infection. Does healing from this type of disease can rarely return to production, and therefore are often prematurely culled (Corpa et al., 2010; Holliman and Girvan, 1986; Rosell and de la Fuente, 2009; Zumpt, 1976).

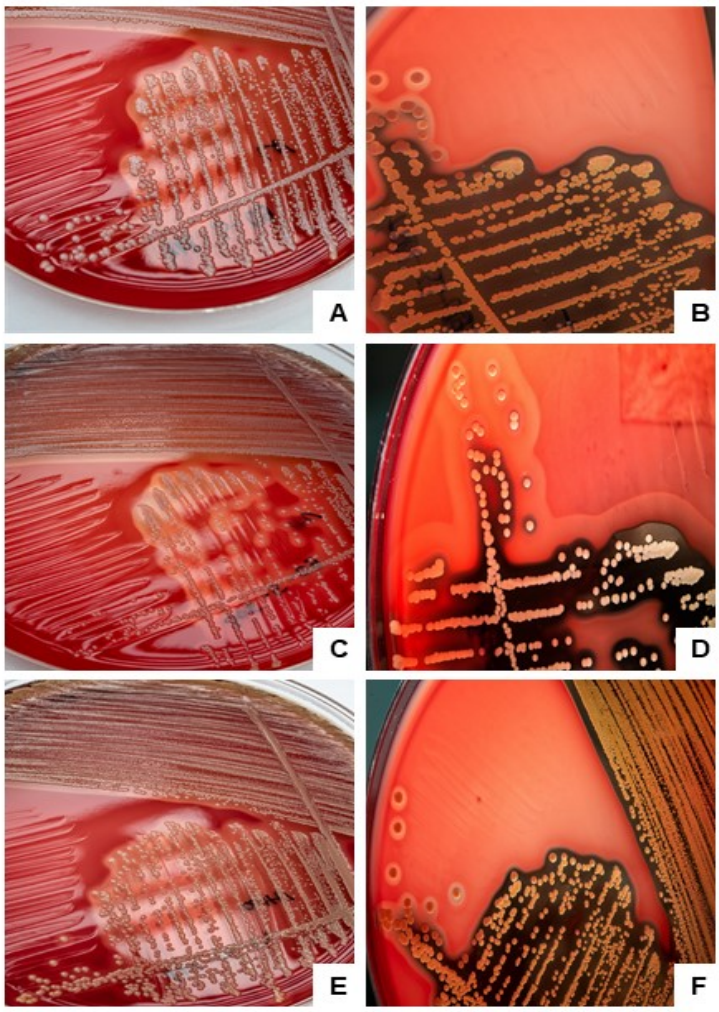
Purulent conjunctivitis, pneumonia, middle ear inflammation, or abscesses in subcutaneous connective tissue can occur at any age (Boucher and Nouaille, 2002; Corpa et al., 2010; Vetési, 1990).



6. Figure: ***Purulent metritis in a rabbit doe****. (The photo is the author's own property.)*

2.3.1. Diagnosis, virulence types

The high rate of typical clinical and pathological symptoms within the herd rises a strong suspicion of the presence of staphylococcosis. The pathogen is easily detectable by routine bacterial cultivation from the organs showing typical lesions. The isolates should be cultured in blood agar plates, under aerobic conditions, incubated at 37 °C. After 18-24 hours, round, shining, convex, usually yellowish carotenoid pigment producing, 1-3 mm diameter colonies are formed, with a typical double haemolytic zone visible around. Bacterial colonies are catalase-positive, oxidase-negative, coagulase-positive, appearing as Gram-positive grape-like clustered cocci in stained smears produced from colonies Figure 5).



7. Figure: ***Blood agar plates inoculated with low virulence (A , B), high virulence (C, D) and atypical high virulence (E, F) Staphylococcus aureus strains, in reflectedillumination (A, C, E) and translucent light (B, D, F). The high virulence strains show beta-hemolysis, the atypical high virulence and low virulence strains are indistinguishable by visual inspection of the colonies.***
(The photos are the author's own property.)

In differential diagnostics one other organism have a significant role. *Pasteurella multocida* is also able to cause dermatitis, mammary gland inflammation, nasal inflammation, pneumonia, metritis or even septicaemia. Differentiation is not a problem, as it typically causes intense loss only by lung disease. In addition, *Pasteurella multocida* in the blood agar forms pinhole, round, shiny, grayish-white colonies with no visible haemolytic zone. *Pasteurella multocida* colonies are catalase and oxidase positive, showing Gram-negative coccoid forms or short rods.

Rabbit specific *Staphylococcus aureus* strains can be divided into variants with low virulence (LV) and high virulence (HV) (Devriese et al., 1996). This classification was introduced based on practical clinical experience, without detailed knowledge of phenotypic or genotypic characterisation of the agent. LV strains infect a small number of individuals, typically the elderly, weakened breeding animals are affected, and the diseases are sporadic (Corpa et al., 2010; Holliman and Girvan, 1986; Okerman et al., 1984). On the other hand, epidemic caused by a HV strain is characterized by high mortality of suckling animals. Also the disease of young breeding animals is typical. According to the generally accepted opinion, these strains are transmitted with asymptomatic breeding animals imported from genetic centres (Corpa et al., 2010; Devriese et al., 1996). The pathogen can be easily detected from the diseased organs of the affected animals (Hermans et al., 2000a). It is very unusual that a virulent strain of a pathogen can be carried by apparently healthy individuals for a long time, but the current knowledge about this pathogen results this as the most probable scenario.

A research team at the University of Ghent conducted infectious experiments with strains of different virulence. Groups of rabbits free of *Staphylococcus aureus* were nasally inoculated with 10^9 colony-forming unit (CFU) pathogens of two LV and HV strains. In the days following the pathogen in all groups was detectable from different areas of the body (nose, ears, legs, abdomen, perianal area). The rate of infection was rapidly reduced in the group infected with LV strains, and the pathogen was no longer be detected after day 5 post inoculation. Although a small number of animal samples (8-17%) inoculated with the LV strain showed the presence of *Staphylococcus* used for the experiment, no symptoms or lesions were detected. In the groups infected with HV strains the number of positive samples was significantly higher, and the rate of reduction of infection was also much lower. At the end of the experiment (28 days), the pathogen was detectable in 87-97% of the animals. Symptoms were also observed in 17% of the animals as small abscesses on their nose, on their feet, and on the perianal area (Hermans et al., 2000a).

In a follow-up experiment (Meulemans et al., 2007), the skin on both sides of the abdomen was aseptically pierced with a tattoo needle, and wounds on one side were inoculated with various strains of *Staphylococcus aureus*. Infections were caused, abscesses formed on the treated areas. In groups challenged with LV strains lesions occurred in 70-80% of the animals. 30-60% of the group showed no symptoms after two weeks, the extent of the lesions did not exceed 7 mm and at the end of the experiment. Only 10-20% of these animals carried the pathogen in infected and non-infected wounds, or in the nasal mucosal cavities. In the HV-challanged groups 90% of the animals developed lesions, all diseased animals failed to fully recover. The extent of the lesions in many cases reached 40 mm, in each case greater than 10-15 mm. At the end of the experiment the pathogen could be detected in all infected areas, 70% of non-infected wounds, and 30% of the animals' nose. Some animals also developed small abscesses on their lips, where the strain used for infection was always present. Statistically significant differences were found in virulence between the LV and HV strains (Meulemans et al., 2007).

The results of the two above-mentioned experiments show that the HV strains are adapted to the rabbit and have pronounced colonizing and infectious ability.

Based on their biochemical properties, LV strains belong to human or poultry types. Each of the HV strains belong to the so-called mixed CV-C biotype. This group produces β-haemolysin and staphylokinase, which is absent in human strains, and, in contrast to poultry-derived strains, forms purple colonies on tryptose agar containing crystal violet. However, these properties cannot be used to separate LV and HV strains, because some LV strains may belong to the mixed CV-C biotype (Corpa et al., 2010; Devriese, 1984; Devriese et al., 1996, 1981).

More accurate identification is possible by typing with an internationally accepted phage set (Parker, 1962). Phage susceptibility of LV strains shows a variety of patterns that could not be systematized due to their diversity (Devriese et al., 1981; Hermans et al., 1999). The HV strains are susceptible to II. 3A, 3C, 66, and 71 phages of the above mentioned set (Viana et al., 2015b). The method allows sure identification, but is slow and cumbersome, thus it cannot be integrated into everyday diagnostics.

The pathogenicity of the *S. aureus* strains is multifactorial and is based on several strategies, such as biofilm formation, adherence, immunomodulation and production of toxins. A more modern approach to characterize apparently different variants is to survey and compare genetic elements related to these microbial phenomena.

Hermans et al. aimed to elaborate a method instead of biotyping and phage-typing which were used to differentiate between LV and HV strains, but these laborious and time-consuming methods are hard to standardise outside specialised laboratories (Hermans et al., 2000b). RAPD analysis has also been used to characterise *S. aureus* strains in bovine mastitis (Matthews et al., 1994). To find out whether RAPD could be used for the distinction of HV *S. aureus* 53 rabbit and 4 human strains were used. Based on these studies, HV strains clearly show a pattern that can be distinguished from the pattern of LV and other *Staphylococcus aureus* strains. The results showed that with the constant use of a positive control HV strain the RAPD-typing can be a useful method as a preventive diagnostic (Hermans et al., 2000b).

Viana et al. investigated *S. aureus* isolates from Spanish rabbit flocks to determine the virulence genes by PCR and Southern blot and to explore the relationship between genotypes and the type of lesions caused by the pathogen (Viana et al., 2015b). By the analysing of the 40 virulence genes in the 69 isolates they concluded that 9 virulence determinants were positive in all *S. aureus* samples. Seven out of the nine were adhesins, one virulence factor was a toxin (hlgC) and one was a protease (sspA). They also find that 11 factors couldn't been detected with the available primers. The rest of the virulence factors were more variable between the analysed samples. The study showed that the type of the lesion could not be related to any combination of the virulence factors. ST121, ST96 and ST2951 were found at the isolates with MLST analysis, all of these sequence types were previously described in rabbit. The variability of the virulence factors can related to new types of mobile genetic elements that can promote genetic diversity and adaptation (Viana et al., 2015b).

The evolution of specialized diagnostics resulted in a multiplex PCR method, which can be used to differentiate between strains of high virulence (HV) and low virulence (LV), and also to identify atypical highly virulent (aHV) strains (Vancraeynest et al., 2007). HV strains have the bbp (bone sialoprotein binding protein) gene (Vancraeynest et al., 2004) and *selm* gene, which is an allele within the *Staphylococcus* enterotoxin gene cluster (Dieter Vancraeynest et al., 2006). Both HV and aHV strains have a nucleotide sequence named flank, which is identified to be specific for virulent strains (Hermans et al., 2001, 2000b). HV, aHV and LV strains are all resulting an amplicon related to the femA gene in this multiplex PCR system. FemA is encoding a factor which is essential for methicillin resistance, and which is universally present in all *Staphylococcus aureus* isolates (Johnson et al., 1991; Mehrotra et al., 2000; Vannuffel et al., 1995).

Vancraeynest et al. evolved a multiplex PCR method suitable for the isolation of HV and LV *Staphylococcus aureus* strains of domestic origin (Vancraeynest et al., 2007). As a positive control, the so-called A femA gene was selected which is a *Staphylococcus aureus* specific gene (Mehrotra et al., 2000; Vancraeynest et al., 2007; Vannuffel et al., 1995). Three genes specific to HV strains and missing from LV strains, bbp, selected from the six genes that make up the *egc* cluster. by the detection of the gene sequence called flank, identified during the RAPD study, between high and low virulence strains. The *egc* cluster encompass six genes: *selo*, *selm*, *sei*, *selu*, *seln* ad *seg*.

Examining the effectiveness of the autovaccination using the above-mentioned experimental infection model, it was concluded that immunization could give partial protection to the animals. The size of the abscesses in the vaccinated rabbits was significantly lower, and the lesion showed a healing tendency. However, vaccination was not able to prevent infection, the development of lesions, or the spread of the pathogen on the body (Meulemans et al., 2011, 2008). Numerous other studies have been conducted to explore the possibilities of immunization against the disease. The efficacy of the various vaccines has been demonstrated in a number of cases under experimental conditions (Adlam and Thorley, 1976; Corpa et al., 2010; Meulemans et al., 2011, 2008), but in practice none of the methods have been fully implemented (Meulemans et al., 2011). Some of the breeders report less damage after treatment, but vaccination-based decontamination has not been successful so far (Corpa et al., 2010).

2.3.2. Epidemiology and treatment

LV strains infect a small number of individuals, typically the elderly, weakened breeding animals are affected, and the diseases are sporadic. If only LV strains are present in a population, then the symptoms occur in a low incidence, so extensive treatment is unnecessary. The culling of symptomatic individuals is a negligible loss. In small-scale operations, or individuals with high genetic value, and for certain forms of disease (sore throat, abscess, nasal inflammation, conjunctivitis), it makes sense to have a specific treatment. Targeted individual antibiotic therapy and, if necessary, surgical treatment (opening and cleaning of abscesses) (Boucher and Nouaille, 2002; Corpa et al., 2010; Vetési, 1990). Most of the low virulence strains are derived from poultry or human biotypes.

HV strains are able to colonize domesticated rabbits permanently, they are proven to be particularly pathogenic for this species (Hermans et al., 2000a; Meulemans et al., 2011, 2007). The HV strains are able to better colonize the rabbit epithelia, and also can cause chronic disease. The international dissemination of such strains has also been described (D. Vancraeynest et al., 2006). HV strains belong to a mixed CV-C genotype, lack the staphylokinase activity and able to synthetize β-haemolysin (Peton and Le Loir, 2014).

Detection of such virulent pathogens can be accomplished by routine bacterial culturing, and a following multiplex PCR to detect three virulence determinant genes (Vancraeynest et al., 2007). Previous studies revealed the existence of highly virulent strains (HV), which were detected in samples derived from farms in Belgium, France, Greece, Italy, Portugal and Spain (D. Vancraeynest et al., 2006). HV strains are identified as Staphylococcus aureus Clonal Complex (CC) type 121 (or ST121), similar strains are known for their increased virulence, and are proven to be globally distributed (Matuszewska et al., 2020). A study revealed that a single nonsynonymous nucleotide mutation in the coding region of an integral membrane protein (dltB) is associated with the host adaptation of CC121 from humans to rabbits (Viana et al., 2015a). Holmes et al. (2016) reported WGS results of Staphylococcus aureus strains isolated from companion rabbits in the UK, one of the isolates belonging to ST121 (Holmes et al., 2016).

Previous studies also reported atypical highly virulent strains (aHV), which were considered virulent based on the severity of the outbreak they caused in Belgium in 1994 (Devriese et al., 1996). With multiplex PCR two virulence genes were not detectable in these strains, so this genotype was considered atypical (Vancraeynest et al., 2007).

Low virulence strains (LV) cause sporadic infections and the prevalence of such strains is difficult to estimate (Hermans et al., 2003). Previous studies found that LV strains have biochemical properties similar to isolates of human or poultry origin (Devriese, 1984).

The flock cannot be released from the disease with the help of hygienic and chemotherapy programs. The culling of symptomatic and suspect animals is even less successful, as new animals introduced in production are quickly infected, and due to their acute clinical illness and mortality, the loss will be even higher (Corpa et al., 2010; Devriese et al., 1996, 1981; Hermans et al., 2003). The only effective means of protection is the culling of the entire infected stock and the introduction of a pathogen free population (Corpa et al., 2010; Hermans et al., 2003; Peton and Le Loir, 2014; Vancraeynest et al., 2007).

On the majority of the visited Hungarian rabbit farms clinical and pathologic evidence of staphylococcis have been found. Unfortunately there was no long term clinical improvement in the affected flocks when treated with antibiotics. According to our own unpublished studies, all *Staphylococcus aureus* strains isolated in rabbit farms are sensitive to tiamulin. In addition, Staphylococci *in vitro* were more susceptible to antibiotic agents that are also toxic to the rabbit (penicillin and its derivatives), or agents with unfavourable kinetics which unable to use systemic treatment (gentamicin).

In 2009, Hungarian studies confirmed the efficacy of bacitracin in the treatment of epizootic enteropathy and a bacitracin-containing medicinal product were registered for rabbits (Német et al., 2010). Thereby, tiamulin treatments used for the same purpose, but also effective against staphylococcosis, have been omitted, and most of the rabbit breeding farms have reported more frequent occurrence of staphylococcosis symptoms and an increase in loss. The complexity of the problem is further illustrated by the fact that tiamulin drug treatment has been associated with suckling rabbits' klebsiellosis - which also confirms that careful consideration of the potential benefits and hazards should always precede the antibiotic treatment of rabbit flocks (Coletti et al., 2001; Német et al., 2011).

3. Aims of the study

Staphylococcosis is a devastating disease in rabbit farming. Production losses due to mortality, premature elimination and slaughterhouse condemnations can result a significant decrease in profitability. Contamination of farms with virulent variants of *Staphylococcus aureus* often calls for radical solutions, usually a complete depopulation and disinfection of the whole unit (Hermans et al., 2003).

The increasing presence of staphylococcosis has been confirmed in Hungarian rabbit farms. Since most of the rabbit flocks contain the *Staphylococcus aureus* bacteria, it is an important question, that the detected pathogen belongs to a group with low or high virulence. The Hungarian stocks are in direct contact with Western European rabbit populations through the trading of breeding animals, so the presence of HV strains in Hungary is very probable.

This study aimed the following:

Ad 1. to implement diagnostic procedures routinely used in West-European rabbit producing countries

Ad 2. to confirm the diagnosis of endemic staphylococcosis based on macroscopic observations on most industrial farms

Ad 2. to collect strains from rabbit industry in Hungary and surrounding countries

Ad 3. to assess the epidemiologic situation about different genotypes of rabbit-pathogenic *Staphylococcus aureus* strains present on Hungarian rabbit farms

Ad 4. to identify symptoms and lesions related to infection with different genotypes, determine organotropism of these variants

Ad 5. to sequence multiple variants and compare it to each other, and also with genomes found in digital repositories

4. Materials and Methods

4.1. Virulence type and tissue tropism of *Staphylococcus* strains originating from Hungarian rabbit farms

4.1.1. Bacterial strains

Staphylococcus sp. strains used in this study were collected from 2009 to 2014, from a total of 30 industrial rabbit farms (Table 1). Twenty-eight of the farms are located in Hungary, one in Austria (XXVI) and the other in Slovakia (XXIV). The doe population of the farms has an average size of 2943 (± 2219 SD; median: 1800, range 300 to 22 000). Thus, this strain collection represents approximately 88300 does and their progeny. In all cases, diseased rabbits were submitted for diagnostic examination to our laboratory by the respective farmers.

1. Table: ***Distribution of examined*** **Staphylococcus** ***strains across farms and virulence types, listed in a decreasing order of the total number of isolations on the farm****. HV: highly virulent, aHV: atypical highly virulent, LV: low virulent,* Staph sp.*: „non-aureus”* Staphylococcus sp.

Farm ID	Doe population	Number of isolates	Virulence type (number of strains)			
			HV	aHV	LV	*Staph. sp.*
VIII	1800	**52**	15	37	0	0
XXII	7000	**52**	0	47	0	5
X	3500	**34**	0	31	1	2
V	4500	**32**	0	31	1	0
XIII	4000	**28**	0	28	0	0
XVIII	6000	**26**	1	25	0	0
XXVIII	1800	**19**	0	19	0	0
XI	4000	**17**	0	11	5	1
XXIII	22 000	**17**	1	16	0	0
XXIX	1200	**17**	0	16	1	0
XXVII	1500	**15**	0	13	2	0
XVI	4000	**9**	1	8	0	0
IX	1600	**7**	4	3	0	0
VII	700	**6**	0	0	3	3
III	700	**5**	0	5	0	0
VI	3000	**5**	0	1	4	0
XX	1500	**5**	0	4	1	0
XV	500	**4**	0	4	0	0
XXX	1800	**4**	0	3	0	1
XIV	700	**3**	0	3	0	0
XXIV	3600	**3**	0	0	3	0
XXVI	300	**3**	0	2	1	0
IV	3000	**2**	0	1	1	0
XII	350	**2**	0	1	1	0
XXV	400	**2**	0	2	0	0
I	700	**1**	0	1	0	0
II	1800	**1**	0	1	0	0
XVII	4000	**1**	0	1	0	0
XIX	2000	**1**	1	0	0	0
XXI	350	**1**	0	1	0	0
		374	**23**	**315**	**24**	**12**

Strains were isolated from infected organs using standard methods (Quinn, 2000). The isolates that were identified as *Staphylococcus* sp. had the following characteristics: Gram-positive, catalase-positive, oxidase-negative cocci with clustered aggregations, forming medium-sized, haemolysing, yellow or grayish pigment producing colonies. Strains were archived at -80°C using standard methods (Markey, 2013).

4.1.2. Multiplex PCR

Extraction of total DNA was performed using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Multiplex PCR was performed using an Eppendorf MasterCycler PCR instrument (Eppendorf, Hamburg, Germany). The primers used for the experiments are shown in Table 2. Typical markers for HV *S. aureus* strains have been selected to investigated with the multiplex PCR. The first target is *bbp*, encoding bone sialoprotein binding protein (Vancraeynest et al., 2004). *Selm* used as a representative for the previously mentioned *egc* cluster. *Flank* derived from a specific RAPD band remarked by Hermans et al. (Hermans et al., 2001). *FemA* gene is specific for *S. aureus* (Vannuffel et al., 1995) and can control the presence of the appropriate quality DNA.

2. Table: **Primers used for multiplex PCR (Vancraeynest et al., 2007)**

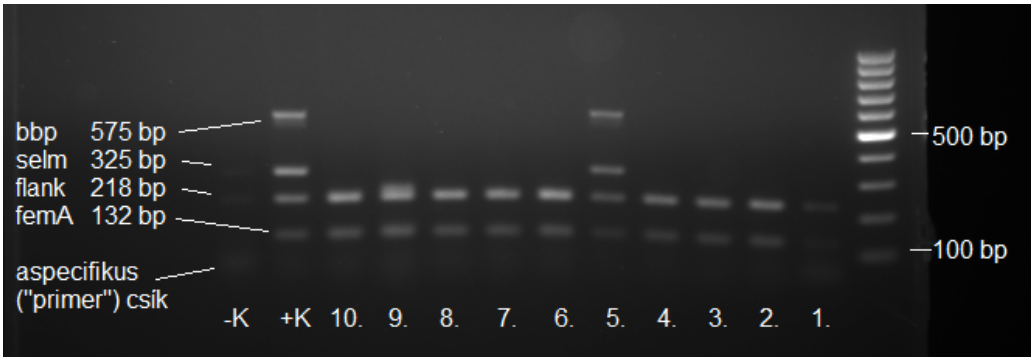
Target	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
bbp	AATTACATCTAGTACTCACAACA	ATGTGCTTGATAACACCATCATC	575
selm	CTATTAATCTTTGGGTTAATGGAAGAAC	TTCAGTTTCGACAGTTTTGTTGTCAT	325
flank	GTAGTCTTTAGATTATTGCCAAC	TCAAGCGTTTGCACTCTGTGG	218
femA	AAAAAAGCACATAACAAGCG	GATAAAGAAGAAACCAGCAG	132

For the PCR reactions, a 30 ul PCR mixture (REDTaq Ready Mix, Sigma) was used contained 6 pmol of the *bbp*, *selm*, *femA* primers and 3 pmol of the *flank* primers with 6 ul DNA sample. The initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 60 s, elongation at 72°C for 60 s. A final elongation added at 72°C for 5 min. After the amplification an electrophoresis was performed in a 1.5% agarose gel with EtBr followed by a visualisation under UV light and photographed. GeneRuler 100 bp DNA Ladder Plus (Fermentas) was used as a molecular weight marker.

A typical HV strain was provided by Katleen Hermans, Ghent University for positive control from the group of specimens used in previous studies, labelled “Sp17”. It is originating from a Spanish rabbit farm with severe staphylococcal mastitis problems. This

strain belongs to the typical highly virulent rabbit *S. aureus* clone, as it shows a mixed CV-C biotype (Devriese, 1984), it is sensitive to phages of phage group II (3A, 3C and 71), it shows the multiplex PCR pattern specific for highly virulent *S. aureus* strains (Vancraeynest et al., 2007), and has pulsed field type N2 and spa type t645 (D. Vancraeynest et al., 2006).

A typical result of the multiple PCR is shown in Figure 7. The HV strains shows all the four amplicons in the assay.



8. Figure: ***Gel eletrophoresis of the amplicons generated by multiplex PCR****.*

4.1.3. Statistical analysis

To facilitate statistical analysis, we have grouped the examined strains into six categories, according to the organ system they originated from. Dermatitis, sore hocks, subcutaneous abscesses and conjunctivitis were grouped into a “cutaneous” (CUT) group. We had fourteen (3.74%) isolates originating from the small intestine of suckling rabbits with diarrhea and lacking any other cultivatable bacteria (e.g. E. coli) in their samples. We decided to separate this group for the sake of analysis as “intestinal” (INT). However, such infection is not described in other sources, the intestinal pathogenicity of these strains was not proven with experiments, and the intestinal presence of the bacteria could be explained by the mastitis of the mother of the examined kits. Mastitis (MAST) and metritis (MET) cases were enrolled into separate groups. The respiratory disease (RESP) group was made of pneumonia and pleuropneumonia cases. Cases of splenitis, hepatitis, polyserositis without pneumonia, peritonitis, arthritis and meningitis were all grouped into “septicaemia and bacteraemia” (SEPT).

Correspondence analysis and association plots were utilized to illustrate associations between virulence type and tissue tropism.

A joint plot figure contains both row and column variables. The scalar multiplication of these vectors is the data value of the corresponding cell of the table. The ellipsoid

outlines indicate the 95% confidence interval (Greenacre, 2007). An association plot can be built on a two-dimensional contingency table containing frequency data. The figures are based on the Pearson's chi-square test, which examines the differences between the observed and predicted frequencies. The Cohen-Friendly association figure represents all cells of the table as rectangles. The area of the rectangle is proportional to the difference between the observed and the expected frequency values. The rectangles in each row are placed above or under a baseline, showing whether the data in the cell indicate a greater or smaller observed frequency compared to the expected value, respectively. Significant differences are marked by colour as well (blue for greater and red for smaller). The number of cases is shown in the boxes. The associations suggested by the illustrative plots were confirmed by Fisher's exact test.

All statistical analyses were conducted using the R 2.12.0 statistical software (Ihaka and Gentleman, 1996). The correspondence analysis was conducted using the package 'anacor', while for the association plot the package 'vcd' was used. For significance level $p<0.05$ was used.

4.2. Sequencing whole bacterial genomes

4.2.1. Draft genome sequence of a highly virulent rabbit *Staphylococcus aureus* strain

A typical HV strain identified as "Sp17", originating from a Spanish rabbit farm with severe staphylococcal mastitis problems, was used for WGS sequencing. This strain belongs to the typical highly virulent rabbit *S. aureus* clone, as it shows biotype mixed CV-C (Devriese, 1984), is sensitive to phages of phage group II (3A, 3C and 71), shows the multiplex PCR pattern specific for highly virulent *S. aureus* strains (Vancraeynest et al., 2007), and has pulsed field type N2 and spa type t645 (D. Vancraeynest et al., 2006).

Total DNA of the strain was subjected to 2×300-bp paired-end Illumina MiSeq sequencing at the Department of Biochemistry, Faculty of Medicine, University of Szeged, Hungary. A total of 3.96 million read pairs were recorded, the estimated coverage of the whole genome is 700×.

The estimated coverage of the subsets of reads was adjusted to 30×, and were assembled de novo using MIRA version 4.0.2 ("Genome Sequence Assembly Using Trace Signals and Additional Sequence Information," n.d.), A5 pipeline version 20130326 (Tritt et al., 2012) and SeqMan NGen version 4.1.2 (DNAStar version 10, DNASTAR Inc., Madison, WI, USA). Scaffolds were built from different assemblies using Mauve version

2.3.1 (Darling et al., 2010) as Geneious version 8.1.2 (Kearse et al., 2012) plugin. This resulted in a total of 10 scaffolds containing 2.684.832 nucleotides. The average G+C content is 32.7%.

Scaffolds were submitted to the RAST annotation server (Aziz et al., 2008). The taxon was set to "*Staphylococcus aureus*" (1280.2034), the genetic code to "11 (Archaea, Bacteria)", the annotation scheme to "ClassicRAST" and both "preserve gene calls", "automatically fix errors", "fix frameshifts" and "backfill gaps" to "no". We have obtained 2395 annotated genes, 50 tRNAs and 5 rRNAs.

Search for similar sequences deposited in GenBank was conducted using BLAST. Best match was *Staphylococcus aureus* subsp. *aureus* 21337 (NZ_JHPZ00000000.1). Pairwise alignment showed 98,56% similarity of these sequences, which indicates close relationship between these strains.

A detailed analysis of virulence genes in these sequences will be conducted to identify key elements responsible for the remarkable pathogenicity of this strain, in order to facilitate the development of effective treatment methods and/or preventive measures against HV *Staphylococcus aureus* infections in rabbits.

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LBCS00000000. The version described is version LBCS01000000.

4.2.2. Draft genome sequence of an atypical highly virulent rabbit *Staphylococcus aureus* strain

An isolate, originating from a subcutaneous abscess of a fattener rabbit, with typical phenotype amongst Hungarian commercial rabbit farm *Staphylococcus aureus* strains, identified as "380/11", was used for WGS sequencing. This strain shows the multiplex PCR pattern specific for aHV *S. aureus* strains (Vancraeynest et al., 2007). This genotype was rarely isolated from diseased rabbits earlier, but currently is the most prevalent genotype at Hungarian commercial rabbit farms (Német et al., 2016).

Total DNA was subjected to 2×300-bp paired-end Illumina MiSeq sequencing at the Department of Biochemistry, Faculty of Medicine, University of Szeged, Hungary. A total of 3.64 million read pairs were recorded, the estimated coverage of the whole genome is 700×.

The estimated coverage of the subsets of reads was adjusted to 30×, and were assembled *de novo* using MIRA version 4.0.2 ("Genome Sequence Assembly Using Trace

Signals and Additional Sequence Information," n.d.), A5 pipeline version 20130326 (Tritt et al., 2012) and SeqMan NGen version 4.1.2 (DNAStar version 10, DNASTAR Inc., Madison, WI, USA). Scaffolds were built from different assemblies using Mauve version 2.3.1 (Darling et al., 2010) as Geneious version 8.1.2 (Kearse et al., 2012) plugin. This resulted in a total of 15 scaffolds containing 2.631.087 nucleotides. The average G+C content is 32.7%.

Scaffolds were submitted to the RAST annotation server (Aziz et al., 2008). The taxon was set to "*Staphylococcus aureus*" (1280.2034), the genetic code to "11 (Archaea, Bacteria)", the annotation scheme to "ClassicRAST" and both "preserve gene calls", "automatically fix errors", "fix frameshifts" and "backfill gaps" to "no". We have obtained 2567 annotated genes, 52 tRNAs and 11 rRNAs. Total CDS is 2.295.057 bases, which is 87.22% of all nucleotides.

A comperative analysis of virulence genes will be conducted in order to evaluate the relations between typical and atypical highly virulent rabbit *Staphylococcus aureus* strains.

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LYXH00000000.

4.3. Comparative analysis of draft genome sequences

Scaffolds from both assemblies were submitted to the RAST annotation server (Aziz et al. 2008). The taxon was set to "*Staphylococcus aureus*" (1280.2034), the genetic code to "11 (Archaea, Bacteria)", the annotation scheme to "ClassicRAST" and both "preserve gene calls", "automatically fix errors", "fix frameshifts" and "backfill gaps" to "no".

For further analysis we used Geneious 10.2.3. (Kearse et al. 2012). Alignment of the two draft genomes was performed using Mauve 2.3.1 plugin (Darling et al. 2010), using whole genome sequence of *Staphylococcus aureus* strain "RF122" (DDBJ/EMBL/GenBank AJ938182) as reference backbone. As our comparison focused on presentation of scaffolds in this paper does not impair the scientific value of the results. All percentages were calculated on the level of nucleotides.

4.4. Genomic analysis of Staphylococcus strains originating from Hungarian rabbit farms

4.4.1. Bacterial strains

We selected 15 HV, 15 aHV, 12 LV and 8 *Staphylococcus* sp., a total of 50 isolates for WGS. One HV isolate was provided for PCR positive control by Katleen Hermans (Ghent University). Digital archives provided another 14 rabbit-originated *S. aureus* genome sequences . Detailed information about the strains used in this study is presented in Table 3.

Table 3. Staphylococcus strains of rabbit origin investigated with whole-genome sequencing. Place of origin is provided, where only the country is available, it is in all capital letters.

	ID	Accession	Year	Species	Origin	Isolation site	Virulence type	SpaType	MLST
1	120-1-09	ERS4418242	2009	Staphylococcus aureus	Dabas	cornea	LV	Unknown	N/A
2	131-1-10	ERS4418234	2010	Staphylococcus aureus	Vaskút	metritis	LV	t1190	publicST2855
3	160-6-09	ERS4418241	2009	Staphylococcus aureus	Hetényegyháza	pneumonia	LV	Unknown	N/A
4	203-2-10	ERS4418236	2010	Staphylococcus aureus	Bükkösd	dermatitis	LV	t127	publicST1
5	203-3-10	ERS4418237	2010	Staphylococcus aureus	Bükkösd	dermatitis	LV	t127	publicST1
6	218-2-09	ERS4418240	2009	Staphylococcus aureus	Mezőtúr	otitis media	LV	t4022	publicST2855
7	326-5-14	ERS4418235	2014	Staphylococcus aureus	Losonctámasi	pododermatitis ulcerosa	LV	t1190	publicST2855
8	418-4-09	ERS4418230	2009	Staphylococcus aureus	Kartal	naval abscess	LV	t11218	publicST2855
9	567-8-11	ERS4418238	2011	Staphylococcus aureus	Fülöpháza	pododermatitis ulcerosa	LV	t2802	publicST96
10	739-4-12	ERS4418233	2012	Staphylococcus aureus	Püspökhatvan	dermatitis	LV	t11218	N/A
11	787-4-11	ERS4418231	2011	Staphylococcus aureus	Galgamácsa	spleen	LV	t11218	publicST2855
12	787-5-11	ERS4418232	2011	Staphylococcus aureus	Galgamácsa	pneumonia	LV	t11218	publicST2855
13	026-1-13	ERS4418208	2013	Staphylococcus aureus	Kardoskút	pododermatitis ulcerosa	aHV	t4770	publicST5993
14	040-8-12	ERS4418206	2012	Staphylococcus aureus	Magfa	pneumonia	aHV	t4770	publicST5993
15	045-2-12	ERS4418201	2012	Staphylococcus aureus	Vitka	internal abscess	aHV	t4770	publicST5993
16	078-13-12	ERS4418211	2012	Staphylococcus aureus	Györköny	pododermatitis ulcerosa	aHV	t4770	publicST5993
17	160-17-9	ERS4418200	2009	Staphylococcus aureus	Hetényegyháza	internal abscess	aHV	t4770	publicST5993
18	235-1-13	ERS4418197	2013	Staphylococcus aureus	Ócsa	mastitis	aHV	t2407	publicST5993
19	235-2-13	ERS4418198	2013	Staphylococcus aureus	Ócsa	mastitis	aHV	t2407	publicST5993
20	360-1-13	ERS4418209	2013	Staphylococcus aureus	Györköny	mastitis	aHV	t4770	publicST5993
21	394-2-12	ERS4418207	2012	Staphylococcus aureus	Vitka	mastitis	aHV	t4770	publicST5993
22	683-7-11	ERS4418202	2011	Staphylococcus aureus	Kartal	spleen	aHV	t4770	publicST5993
23	686-7-13	ERS4418210	2013	Staphylococcus aureus	Alsótold	pneumonia	aHV	t4770	publicST5993

24	722-7-11	ERS4418203	2011	Staphylococcus aureus	Hetényegyháza	pododermatitis ulcerosa	aHV	t4770	publicST5993
25	726-1-12	ERS4418212	2012	Staphylococcus aureus	Püspökhatvan	abscess	aHV	t711	publicST5993
26	751-2-13	ERS4418199	2013	Staphylococcus aureus	Ocsa	mastitis	aHV	t2407	publicST5993
27	787-10-11	ERS4418205	2011	Staphylococcus aureus	Galgamacsa	pneumonia	aHV	t4770	publicST5993
28	226-3-13	ERS4418221	2013	Staphylococcus aureus	Györköny	liver	HV	t645	publicST121
29	226-4-13	ERS4418222	2013	Staphylococcus aureus	Györköny	arthritis	HV	t645	publicST121
30	226-6-13	ERS4418227	2013	Staphylococcus aureus	Györköny	arthritis	HV	t645	publicST121
31	226-7-13	ERS4418228	2013	Staphylococcus aureus	Györköny	arthritis	HV	t645	publicST121
32	231-4-14	ERS4418224	2014	Staphylococcus aureus	Györköny	internal abscess	HV	t645	publicST121
33	231-5-14	ERS4418225	2014	Staphylococcus aureus	Györköny	spleen	HV	t645	publicST121
34	306-5-09	ERS4418214	2009	Staphylococcus aureus	Cegléd	pneumonia	HV	t645	publicST121
35	630-6-12	ERS4418219	2012	Staphylococcus aureus	Magfa	pneumonia	HV	t645	publicST121
36	655-4-11	ERS4418216	2011	Staphylococcus aureus	Harta	dermatitis	HV	t645	publicST121
37	655-6-11	ERS4418217	2011	Staphylococcus aureus	Harta	dermatitis	HV	t645	publicST121
38	655-7-11	ERS4418218	2011	Staphylococcus aureus	Harta	internal abscess	HV	t645	publicST121
39	655-8-11	ERS4418226	2011	Staphylococcus aureus	Harta	spleen	HV	t645	publicST121
40	699-8-12	ERS4418220	2012	Staphylococcus aureus	Györköny	metritis	HV	t645	publicST121
41	709-7-13	ERS4418223	2013	Staphylococcus aureus	Alsótold	internal abscess	HV	t645	publicST121
42	773-1-10	ERS4418215	2010	Staphylococcus aureus	Ócsa	mastitis	HV	t645	publicST121
43	SP17	LBCS00000000	2004	Staphylococcus aureus	SPAIN	mastitis	HV	t645	publicST121
44	574-2-10	ERS4418243	2010	Staphylococcus saprophyticus	Nemerőpuszta	-	Staph sp.	-	-
45	574-3-10	ERS4418244	2010	Staphylococcus saprophyticus	Nemerőpuszta	-	Staph sp.	-	-
46	574-4-10	ERS4418245	2010	Staphylococcus saprophyticus	Nemerőpuszta	-	Staph sp.	-	-
47	574-7-10	ERS4418246	2010	Staphylococcus sp.	Nemerőpuszta	-	Staph sp.	-	-
48	574-8-10	ERS4418247	2010	Staphylococcus saprophyticus	Nemerőpuszta	-	Staph sp.	-	-
49	633-1-10	ERS4418248	2010	Staphylococcus xylosus	Nemerőpuszta	-	Staph sp.	-	-
50	633-2-10	ERS4418249	2010	Staphylococcus cohnii	Nemerőpuszta	-	Staph sp.	-	-
51	633-3-10	ERS4418250	2010	Staphylococcus xylosus	Nemerőpuszta	-	Staph sp.	-	-
52	-	ERR387096	2013 April	Staphylococcus aureus	Manchester 13/1	-	-	t440	publicST30
53	-	ERR387097	2013 April	Staphylococcus aureus	Manchester 13/2	-	-	t440	publicST30
54	-	ERR387166	2013-June	Staphylococcus aureus	ENGLAND	Ventral vulva abscess	-	Unknown	publicST3126
55	-	ERR387196	1999	Staphylococcus aureus	SCOTLAND 99	Sub-cutaneous abscess	-	t645	publicST121
56	-	ERR387257	2013-June	Staphylococcus aureus	ENGLAND 06.13/2	Skin infection	-	Unknown	publicST15
57	-	ERR425000	2007-June	Staphylococcus aureus	SPAIN 06.07/1	-	-	-	publicST121
58	-	ERR425012	2003	Staphylococcus aureus	SPAIN 03	-	-	t645	publicST121
59	-	ERR425013	2010	Staphylococcus aureus	ITALY	-	-	t645	publicST121
60	-	ERR494744	2009-Sept	Staphylococcus aureus	Stirlingshire	Nasal sample post mortem	-	-	publicST3092
61	-	ERR494745	2012-June	Staphylococcus aureus	Glasgow 12/1	Chest cavity abscess	-	t15409	publicST39
62	-	ERR494746	2012-June	Staphylococcus aureus	Glasgow 12/2	Lower jaw abscess	-	t1977	publicST2257

4.4.2. Isolation of DNA and library preparation

Genomic DNA from the pure culture of isolates was isolated using the NucleoSpin Microbial DNA Kit (Macherey-Nagel) according to the manufacturer's instructions. The quality and quantity of the isolated 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 v3 using paired end 300bp reads. The coverage was 103.0-246.0, with an average of 156.6.

4.4.3. Bioinformatic analysis

The fastq files were imported directly from Illumina BaseSpace to the BioNumerics software's (version 7.6) (Applied Maths, Belgium) cloud-based calculation engine. De novo sequence assemblies were made with the SPAdes de novo genome assembler (version 3.7.1) (Nurk et al., 2013). The raw reads (fastq files) and the de novo assembled genome of each isolate were submitted to the BioNumerics' S. aureus wgMLST scheme for assembly-free (AF) and assembly-based (AB) wgMLST allele calling. The wgMLST scheme contains 3897 wgMLST loci and 7 MLST loci, the allele sequences are matched with a database, and each one is translated into a single numerical value. MLST and spa typing was also performed within the software package. A circular dendrogram was created in the Advanced cluster analysis function to help understanding the results. All sequencing data are available in ENA under the project number PRJEB37661 and ERR4017499-ERR4017552 individual accession numbers.

For annotation of draft genomes, we used RASTtk (Brettin et al., 2015) and similar genomes were identified using the Similar Genome Finder service of PATRIC 3.6.3 (Wattam et al., 2017).

MLST and spa types were confirmed by PCR and Sanger sequencing from multiple isolates from each genotype.

5. Results

5.1. Virulence type and tissue tropism of *Staphylococcus* strains originating from Hungarian rabbit farms

5.1.1. Summary of multiplex PCR results

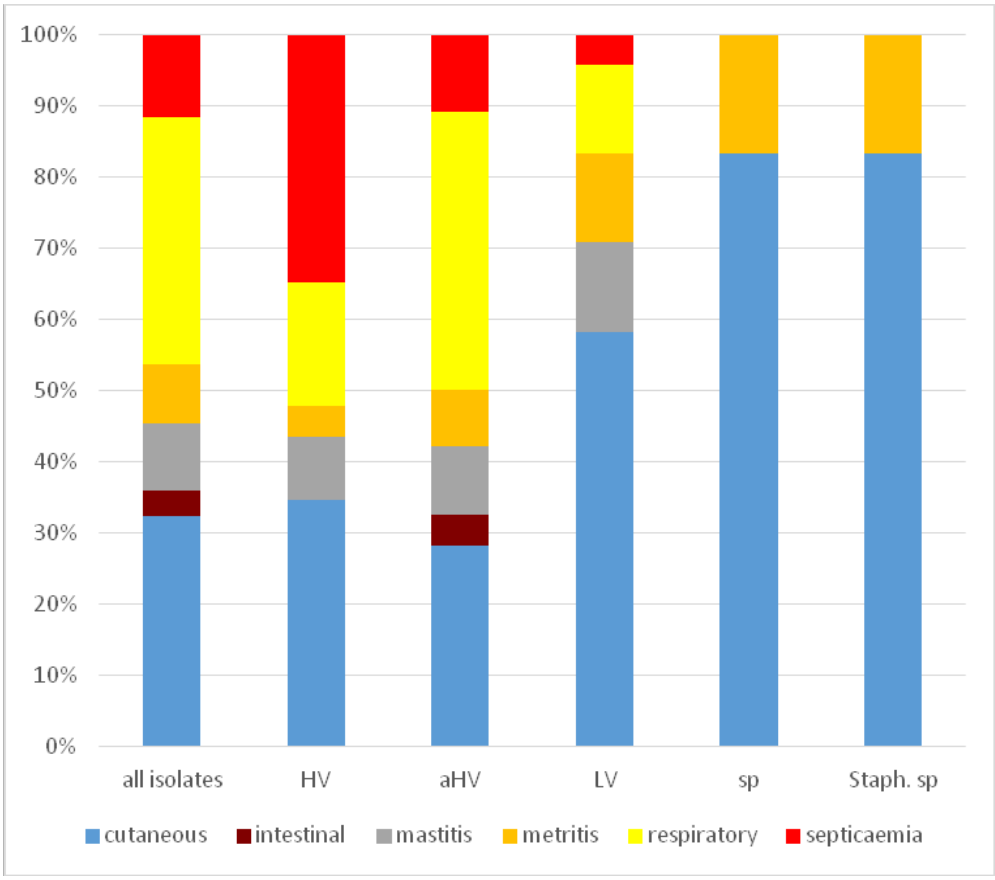
The majority of the strains (84.2%, n=315) belonged to a previously rarely isolated aHV type. We detected 23 (6.1%) HV strains. In cases when more than one strain was isolated from one outbreak, HV and aHV strains were regularly detected simultaneously from different animals with similar lesions. HV isolates were found on only six farms (20% of all examined farms), 15 (65%) of them originating from a single farm. Only 30% of all strains (n=50) isolated from this farm were of the HV genotype. The isolates from 2009 and 2010 had only one HV strains each year (1,0 and 1,9% respectively). The strains from 2011 and 2012 had 4 (7,1%) and 3 (5,1%) HV genotypes, respectively. In 2013 we isolated 12 HV strains (16,9%). In 2014, due to the depopulation of some contaminated farms, the number of HV strains decreased to 2 (5,9%). The percentage values are calculated with the total number of isolations from that year. Twenty-five strains (6.7%) were negative for all three virulence-related genes of this PCR-system, thus they were classified as low virulence (LV) strains. Surprisingly, 12 strains (3.2%) identified on morphological and biochemical grounds as *Staphylococcus aureus* were also negative for the *femA* gene. These strains were labelled as *Staphylococcus* sp. Detailed results of the multiplex PCR are shown in Table 4.

4. Table*: **Organotropism of different virulence types** (number and percentage of strains, n=374). HV: highly virulent, aHV: atypical highly virulent, LV: low virulent, Staph sp.: „non-aureus" Stapylococcus sp.*

Organ system		all strains		HV		aHV		LV		Staph. sp	
		no.	%	no.	%	no.	%	no.	%	no.	%
Cutaneous	CUT	121	32,4	8	34,8	89	28,3	14	58,3	10	83,3
Intestinal	INT	14	3,7	0	0	14	4,4	0	0	0	0
Mastitis	MAST	35	9,4	2	8,7	30	9,5	3	12,5	0	0
Metritis	MET	31	8,3	1	4,3	25	7,9	3	12,5	2	16,7
Respiratory	RESP	130	34,8	4	17,4	123	39	3	12,5	0	0
Septicaemia	SEPT	43	11,5	8	34,8	34	10,8	1	4,2	0	0
Total		**374**		**23**		**315**		**24**		**12**	

5.1.2. Summary of statistical analysis

The raw organotropism data of the different genotypes is numerically shown in Table 3 also presented graphically in Figure 7.

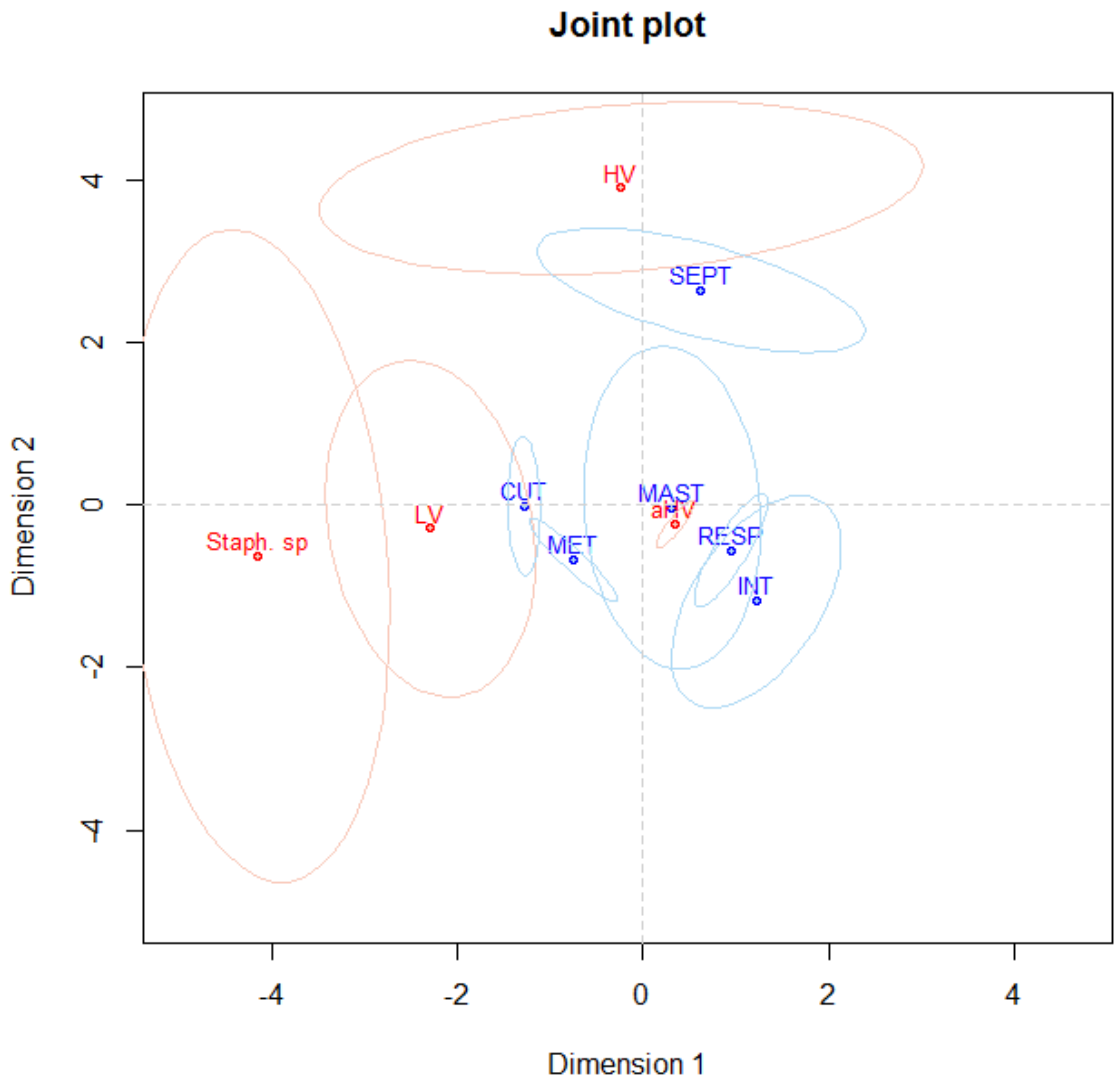


7. Figure: ***Organotropism of* Staphylococcus *isolates****. HV: highly virulent, aHV: atypical highly virulent, LV: low virulent, Staph sp.: „non-aureus"* Staphylococcus sp.

Strains were isolated most frequently from respiratory (34.8%) and cutaneous (32.4%) lesions. Septicaemia and bacteraemia related diseases ("SEPT" group) were presented roughly by the same percentages as mastitis and metritis (11.5%, 9.4% and 8.3%, respectively). The intestinal presence of the pathogen was diagnosed in only 3.7% of all cases, and only in the aHV genotype group.

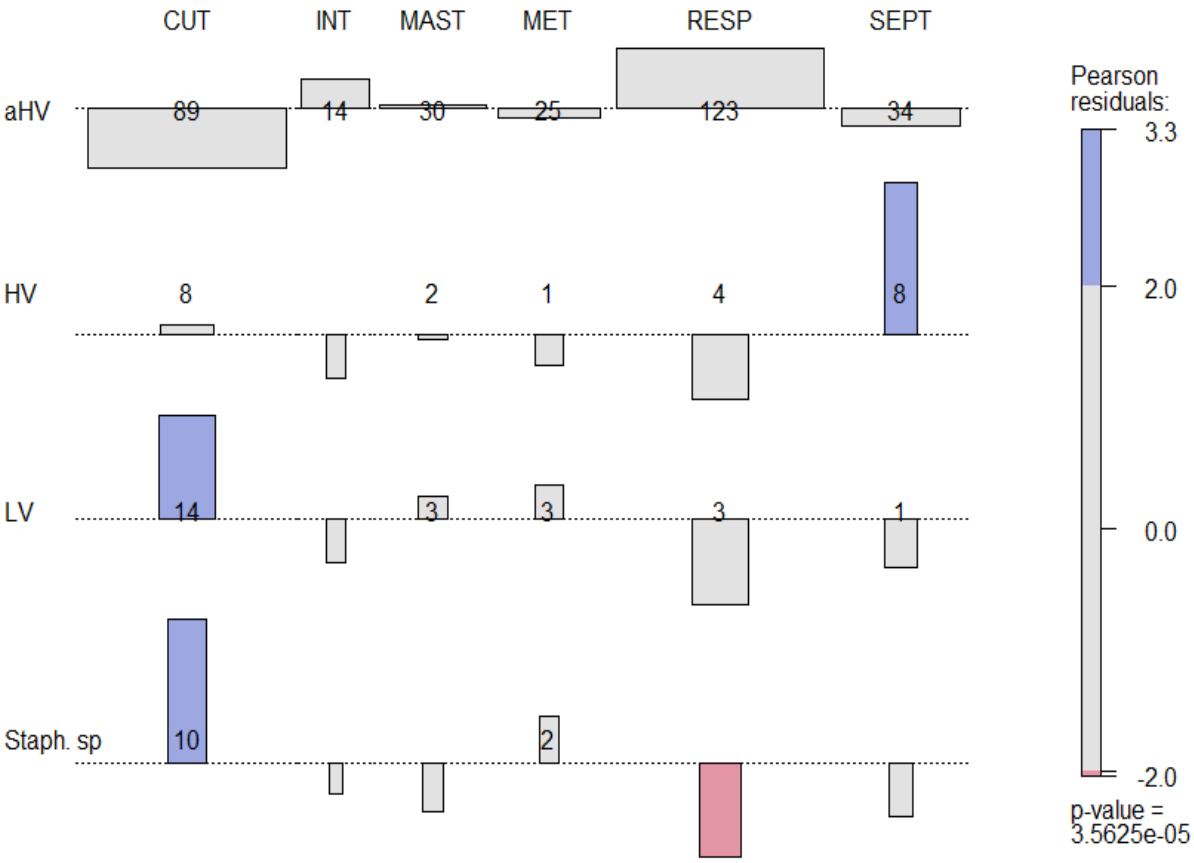
HV strains were substantially over-represented in the "SEPT" category. As aHV strains gave 84.2% of all specimens, the organotropism of this genotype is very similar to the distribution presented for all strains. As expected, the less virulent LV and "*Staph. sp*." strains were mostly isolated from cutaneous lesions. LV strains were found in all but intestinal organ systems, "non-*aureus*" species were only present in two cases of metritis besides cutaneous lesions.

The joint plot in Figure 8. indicates a separation of “HV” strains, as this is the only group placed in the positive range of the y axis. The confidence intervals of “Staph. sp” and “LV” show some overlap, as these strains had the most similar organotropism. Regarding organ systems, the “SEPT” group is the most separated from the others, as its confidence interval does not overlap with any other category. Considering the connection between virulence type and organotropism, the geometrical proximity indicates a connection between “HV” and “SEPT” and between “aHV” and “MAST”.



8. Figure: ***Joint plot of organotropism and virulence types. The ellipsoid outlines indicate the 95% confidence interval****. HV: highly virulent, aHV: atypical highly virulent, LV: low virulent , Staph sp. : „non-aureus” Staphylococcus sp., CUT: cutaneous, INT: intestinal, MAST: mastitis, MET: metritis, RESP: respiratory, SEPT: septicaemia*

As a second approach, we made an association plot for an alternative graphical representation of the data above (Figure 9). The association plot confirms the connection between “HV” and “SEPT” and indicates that “LV” and “Staph. sp.” groups are more likely to be cultured from cutaneous diseases.



9. Figure: ***Association plot between virulence type and tissue of origin****. HV: highly virulent, aHV: atypical highly virulent, LV: low virulent, Staph sp.: „non-aureus” Staphylococcus sp., CUT: cutaneous, INT: intestinal, MAST: mastitis, MET: metritis, RESP: respiratory, SEPT: septicaemia*

Associations suggested by the illustrative plots were confirmed by Fisher’s exact test. HV strains were present 4.8 times more frequently in septicaemia than in any other lesion/organ (p=0.002, odds ratio: 4.8, 95% confidence interval: 1.6-13). Thirty-five percent of all HV isolates originated from septicaemia, while the average isolation percentage of other virulence types in septicaemia was only 10%. HV strains caused 19% of all septicaemia cases, although only 6.15% of all isolates were categorized as HV.

LV strains were present 3.2 times more frequently in cutaneous lesions than in any other organ (p=0.010, odds ratio: 3.2, 95% confidence interval: 1.3-8.2). Fifty-eight percent of all LV isolates originated from skin lesions, while other virulence types had an average prevalence of 30%.

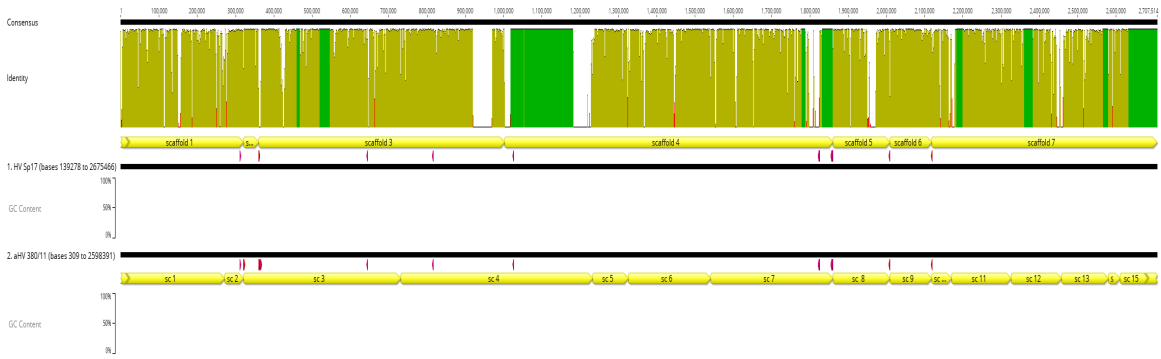
The "*Staph. sp.*" group was present 11.2 times more frequently in cutaneous isolations than from any other organ, but the very wide confidence interval indicates that this outcome is less reliable than the other results (p=0.003, odds ratio: 11.2, 95% confidence interval: 2.4-107.1). Strains belonging to this virulence type were never isolated from respiratory lesions, which was the other most represented group (34.75% of all isolates).

5.2. Comparative analysis of draft genome sequences

For the HV strain the assembly resulted in a total of 10 scaffolds containing 2.684.832 nucleotides, with an average G+C content of 32.7%. We have obtained 2395 annotated genes, 50 tRNAs and 5 rRNAs. The total of coding DNS sequences (CDS) within this assembly is 2.180.424 nucleotides, which means that 81,21% of encoding regions.

Regarding the aHV strain, the same process resulted a total of 15 scaffolds containing 2.631.087 nucleotides, with an average of 32.7% G+C content. We have obtained 2567 annotated genes, 52 tRNAs and 11 rRNAs. Total CDS is 2.295.057 bases, which is 83.69% of all nucleotides.

As a general comparison we aligned the draft genome sequences. The result showed an 88,1% pairwise identity (Figure 10.).

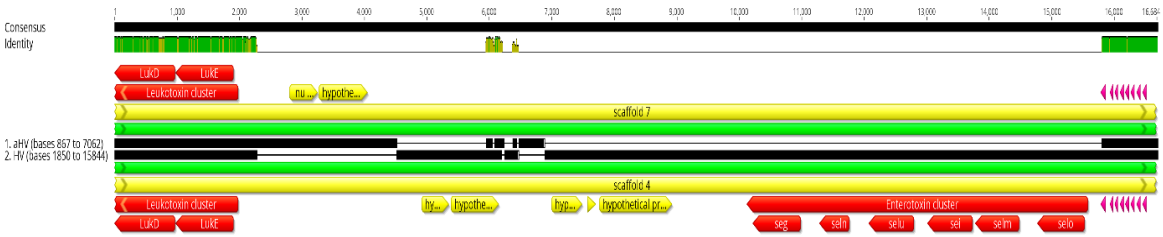


10. Figure: ***General overview of the alignment of the two draft genomes.*** *Scaffold order and orientation was determined arbitrary to facilitate visualisation. (A bigger version of the figure is included in the appendix.)*

The two genomes had several major insertions/deletions, including an approximately 39 kbp phage insertion site in the HV strain and a different 42 kbp phage insertion in another locus in the aHV genome. The two phage insertion sites were somewhat similarly structured regarding major phage component genes, but pairwise nucleotide identity was only 51,8 % between these regions. Numerous insertion/deletion sites with length up to 15 kbps are scattered through the two genomes (Figure A1.).

We compared 63 annotated genes, which are described as major virulence factors of *Staphylococcus aureus* (Chavakis et al., 2007; Vancraeynest et al., 2004; von Eiff et al., 2004). Description of all results can be found in Table A1.

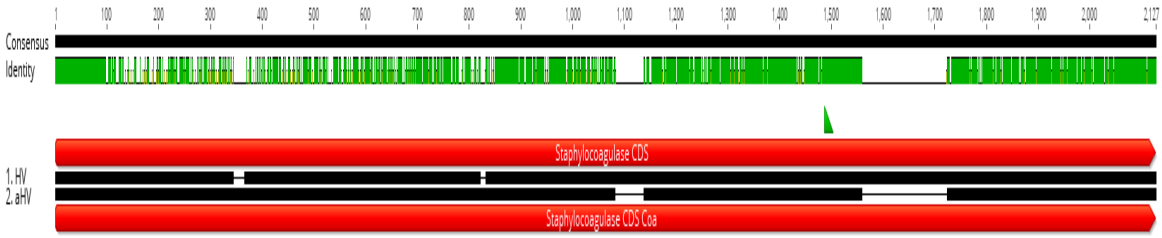
The genes coding for the extracellular enzymes of the two genotypes have very similar nucleotide sequences, with more than 96% similarity for all 22 genes examined. Most of the toxins (α, β, δ-toxins, Panton-Valentine and D, E, M leucocidins) also showed very similar alleles with at least 97% pairwise identity. However, the whole 5,4 kbp superantigen (enterotoxin) cluster was absent in the aHV genome, as a part of a major 13,5 kbp insertion/deletion site (Figure 11.).



*11. Figure:**Enterotoxin gene cluster in the HV strain.** The aHV genome did not have these features. In routine diagnostics the amplification of selm is only possible with HV strains. (A bigger version of the figure is included in the appendix.)*

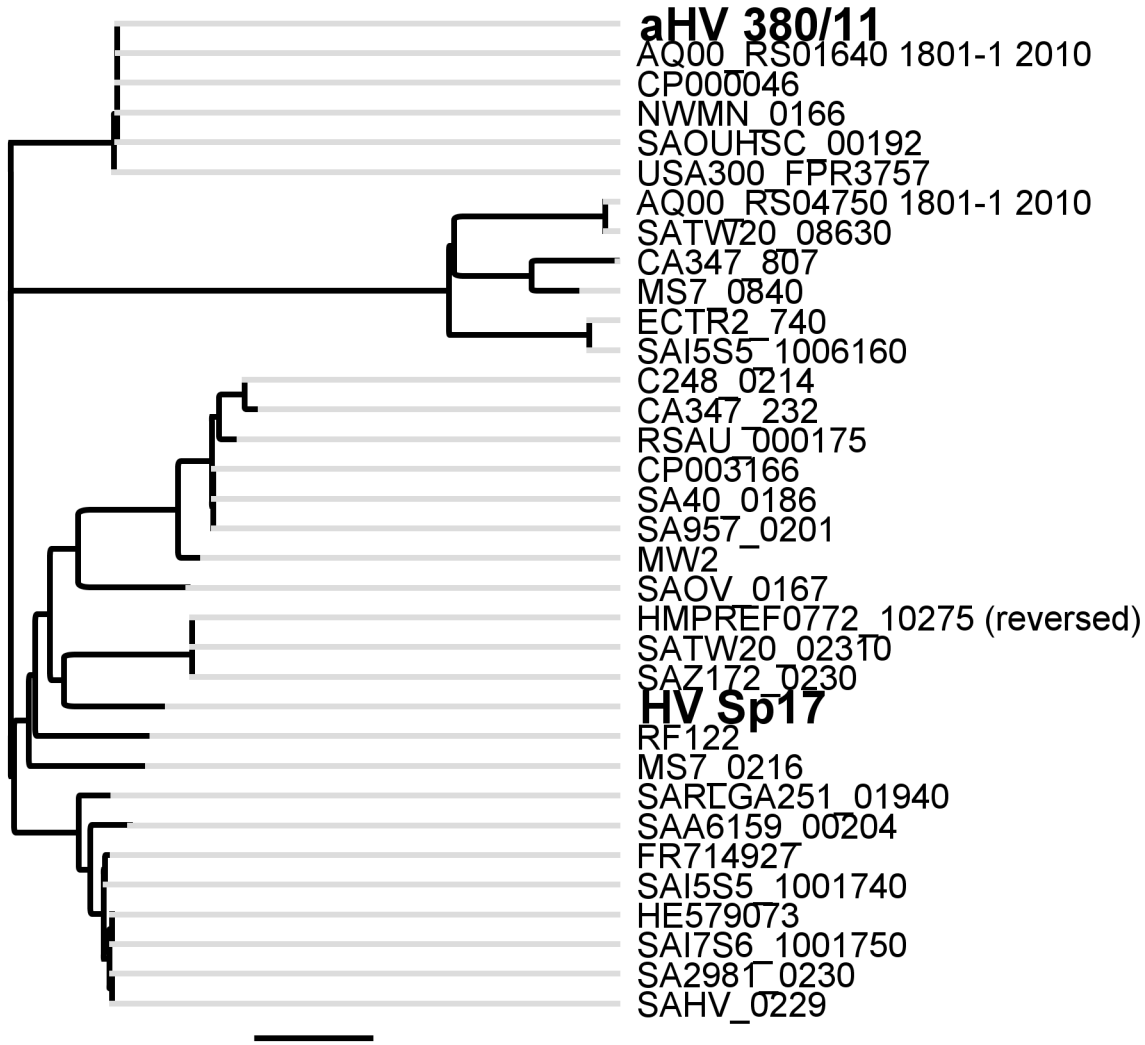
The enterotoxin gene cluster in the HV strain consisted of *selo*, *selm*, *sei*, *selu*, *seln* and *seg* genes, respectively. The multiplex PCR system for virulence determination amplifies the *selm* gene, the sequence in our project contained the described 325 bp amplicon, but the primer binding region contained one C/A mismatch in the forward primer in position 23, and one A/T mismatch in the reverse primer at the last (26th) nucleotide.

More notable genetic variability was found regarding the adhesive molecules of the two strains. Eight out of 23 genes within this group had less than 90% pairwise similarity with each other. The pronounced heterogeneity was found comparing staphylocoagulase (*coa*) genes, 63,9% pairwise identity on the level of nucleotides, and a 71.6% identity when comparing the translation of these genes (Figure 12., Figure A2.).



12. Figure: ***Identity between the two staphycoagulase (coa) sequences.*** *This gene is the least similar amongst the virulence genes examined. (A bigger version of the figure is included in the appendix.*

We used these two sequences with other 25 staphylocoagulase sequences derived from NCBI Genbank to demonstrate the patristic distance between the two rabbit originated variants (Figure 13.). We observed significant differences comparing the coding sequences for fibrinogen binding factors (*FnbA*, *FnbB*, *ClfA*). Collagen binding protein (*cna*), which was described as a major adhesive factor was not found in any of the rabbit-originated sequences, according to previous studies (Hermans et al., 2000b; Holliman and Girvan, 1986). The phylogenetic tree had been created in Geneious Tree Builder using default parameters ((Kearse et al., 2012).



13. Figure: ***The patristic distance between the two rabbit originated variants (HV and aHV).*** *The other staphylococcus coagulase (coa) sequences were derived from NCBI Genbank.*

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5.3. Genomic analysis of Staphylococcus strains

The phylogenetic tree constructed from the wgMLST results of our Staphylococcus aureus strains classified them into four main branches (Figure 14). Four other groups were created from the sequences derived from previous studies. The root is calculated within the dataset, and the branch length and the indicated number shows how many loci are different between neighbours. The clusters have a marked distance (40% of all loci are different) from the calculated root, which was isolated in Scotland in 1998. Some isolates derived from previous studies blend very well into the phylogenetic tree. The groups inferred in the tree are very consistent with the genotype determined by multiplex PCR (HV, aHV, LV) of our isolates.

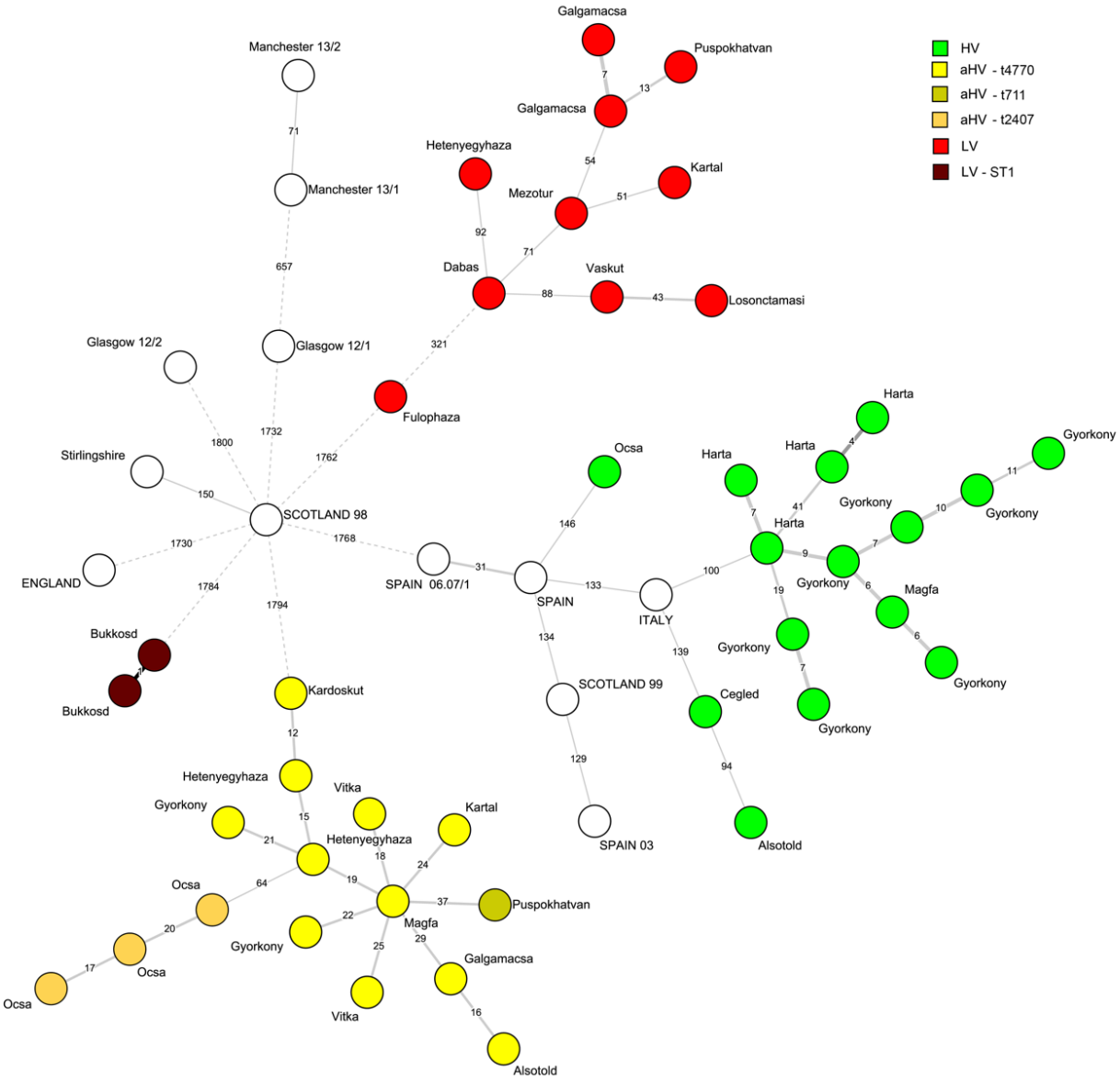
The HV strains, together with one isolate from Scotland, and the strains from Spain and Italy belonged to the ST121 type and a t645 spa type, as reported in previous reports about HV S. aureus strains. The Hungarian isolates were different in less than 0.3%, and other ST121 isolates were also more than 99% identical on the examined loci.

The aHV strains form a similarly homogenous cluster. The wgMLST revealed polymorphism on an average of 21.75 loci, which means 0.55% of the complete feature set. All aHV strains showed a novel MLST combination (ST5993) because of unique gmk and pta sequences. The aHV strains were mostly spa type t4770, and one the very similar t711 type. All these strains originated from a group of farms, where the breeding animals were provided by the same genetic centre. Three strains had the t2407 spa type and these isolates were cultured from diseased rabbits from the Ócsa production unit.

LV strains grouped into two main clusters. 10 out of 12 strains were clustered as close as HV and aHV strains, despite the 5 different MLST patterns and 7 different spa types. Three ST types and two spa types were not identified before. On the other hand, two strains belonging to the very first MLST group (ST1), originating from the same isolated small-scale rural unit (Bükkösd), formed a separate branch.

The strains classified as Staphylococcus sp. were not included in the tree, because wgMLST failed to identify enough loci to be able to classify them. The PATRIC Similar Genome Finder service resulted hits of Staphylococcus saprophyticus, xylosus and cohnii.

Figure 14. Circular dendrogram of Staphylococcus aureus isolates of rabbit origin. Created with BioNumerics 7.6 Advanced cluster analysis, based on the results of whole-genome MLST. The distance between neighbours is the number of loci that have a different allele within the set of 3897 SAUR loci. The length of the branches correlates with the square root of the distance. Colours are used with the strains from the author's collection. Different shades are used to present different Spa or MLST types within a cluster.



6. Discussion

6.1. Virulence type and tissue tropism of *Staphylococcus* strains originating from Hungarian rabbit farms

Staphylococcosis has a major economic impact on Hungarian rabbit farming. Generally, this disease is present on all farms, but epidemic spread can be prevented by management and medication.

Staphylococcosis is a disease having major economic impact on industrial rabbit meat production. Infections caused by highly virulent *Staphylococcus aureus* strains result in severe clinical conditions; these strains are also frequently resistant to antimicrobials. An outbreak of HV strain staphylococcosis hinders profitable production, and frequently necessitates culling the entire flock (Hermans et al., 2003).

Genotypic characterization of *Staphylococcus* strains of rabbit origin has never been performed previously in Hungary. As modern rabbit farming in Hungary is based on French hybrid breeds, the presence of pathogens reported from Western European countries in Hungarian farms was predictable.

The bacterial pathogens isolated from diseased organs are routinely archived for scientific purposes. In a study we reported the result of multiplex PCR performed on n=374 *Staphylococcus* strains, originating from a total of 30 farm units, 28 from Hungary, representing approximately 85% of the Hungarian industrial rabbit population.

6.1.1. Detection of virulent strains

Here, we present a survey on the genetic characterization of *Staphylococcus aureus* from rabbits, a subject that is little investigated. A historic collection of strains mostly originating from the Hungarian rabbit industry was examined for virulence characteristics. In the limited number of studies, which aimed to determine the overall isolation rates of HV *Staphylococcus aureus*, and were publicized before the development of the multiplex PCR method, the reported percentages of contaminated rabbits were 43.3-90.0% and 70.0%, respectively in different flocks (Agnoletti et al., 2008; Hermans et al., 1999). Nine years passed since the development of the multiplex PCR method for virulence type determination, but information about the dissemination of such strains

remained very scarce. The presence of more virulent variants in Hungary was predictable due to the origin of the rabbit populations and the mortality data and diagnostic findings of the farms.

The first striking result of our study was that the majority of the strains (84.0%) belonged to the atypical highly virulent (aHV) genotype. For this genotype only one of the three targeted virulence-related genes can be amplified with the multiplex PCR. Such strains were only detected in a limited number of examined specimens previously, and these strains originated from an isolated case of the disease (Devriese et al., 1996; Vancraeynest et al., 2007). The relation between the aHV strains described before and the strains currently being present in Hungary needs further investigation.

Typical HV strains were detected in low numbers (n=23), and only from a limited number of farms. Previous studies reported that contamination with HV strains would make the complete depopulation and repopulation of the flock necessary. According to one report, many farms contaminated with HV strains were unable to sustain profitable production. Most of them were forced to change the whole flock, or to cease rabbit farming (Hermans et al., 2003).

On three farms (VIII, IX and XVIII) where we isolated HV strains, serious technological predisposing factors, major hygienic and management errors were present. As a consequence, a plethora of other infectious diseases developed. One unit has stopped rabbit farming, while the second is still struggling with unprofitable production. The third farm was completely renovated and repopulated with new flock from the same nucleus flock as before. Since then this farm has been able to sustain normal production, and HV strains have not been isolated again. Further HV isolates came from three farms (XXIII, XVI, XIX) which are not serviced regularly by our laboratory. All these farms had one single HV isolate, being 5.9-100% of all isolates from these flocks within this survey. None of them informed us about any uncontrollable episodes of staphylococcosis, but this information might not be complete. Two of these farms have been able to sustain production until the present day, the third farm was forced to cease production for reasons not related to staphylococcosis.

The first three cases seem to confirm that contamination with HV strains will hinder profitable production. However, more data is needed to generally assess the clinical and economical relevance of HV strain infection of rabbit flocks.

Given its proposed economic significance, the origin of the HV contamination is a crucial question. Pathogens in modern enclosed farms can originate from a few definite sources. Usually their most probable entry route is via incoming infected breeding animals.

All three flocks originated from breeding centres (V, VI, X, XI, XXII), where 140 isolations in 5 years have not yielded a single HV strain. The statistical significance of none of the nucleus farms yielding any HV strain, versus the n=23 (9.8%) HV isolates within the 244 strains from all other farms is obvious (Fisher's exact test, $p<0.001$). If HV strains were introduced to the contaminated farms with asymptomatic young breeders, then the breeding centres should have been contaminated with these strains as well, but they did not cause any epidemic, and our large-scale diagnostic program was unable to detect their presence.

It is possible to transfer *Staphylococcus* strains between farms by humans, materials or equipment. In these cases, this route is not probable, because these farms are separated from each other in terms of geographical location, property relations or production structure. Moreover, other examined units, which would have had a plausible chance to be contaminated from these three flocks via indirect routes, never submitted rabbits infected with HV strains, regardless of dozens of staphylococcosis outbreaks during the examined period.

The third possible route of transmission would be contact with pests or wild animals, but since HV strains are defined as highly adapted and specific to domesticated rabbits, this route does not seem to be more than a theoretical possibility.

As the most probable source of HV strains seems to be the contaminated nucleus flock, it is really intriguing why these strains were not isolated from the units where does of contaminated farms were born and raised. We have no clear explanation for this, however, there are two factors to consider. The first is that severe predisposing factors (inferior hygienic and management conditions etc.), not seen at breeding centres, were present in all three cases. It can be speculated that asymptomatic rabbits may carry HV strains, and only radical stress factors can facilitate the emergence of an epizootic disease caused by such genotypes.

Second, aHV strains were present on all but one farm in this survey (the only exception being farm XIX with one HV isolate from 2009). Also, LV, aHV and HV strains were regularly isolated simultaneously within diagnostic cases from similar lesions, but never from the same animal.

Microorganisms dwelling in upper respiratory organs compete for space and resources. Direct interactions, like competition for adhesion sites, nutrients or other receptor-mediated interactions, and also indirect interactions via secreted factors affecting the competitors or the immune system, were all described before (Bibel et al., 1983; Margolis et al., 2010; Murphy et al., 2009). A negative correlation between nasopharyngeal

colonization of *Staphylococcus aureus* and *Staphylococcus epidermidis* (Iwase et al., 2010), *Streptococcus pneumoniae* (Bogaert et al., 2004; Regev-Yochay, 2004) and *Corynebacterium sp.* (Lina et al., 2003; Yan et al., 2013) were reported previously. A recent study confirmed that nasal commensal *Staphylococcus lugdunensis* strains are producing an antibiotic peptide, which is bactericidal against major pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Zipperer et al., 2016).

It might occur that aHV strains interact somehow with HV strains, either directly, through competition, or indirectly, or even by involving the immune system of the rabbit. It could be that aHV strains are less pathogenic yet more successful in colonizing mucous membranes, so they might constitute the dominant *Staphylococcus* type until remarkable external factors alter this equilibrium.

More data about virulence types of *Staphylococcus* isolates from rabbit farms (preferably from multiple countries), and studies based on simultaneous and parallel experimental infections with multiple genotypes would be needed to gain more knowledge on this phenomenon. These findings also indicate the need for surveying non-diseased animals to detect the prevalence of asymptomatic carrying of aHV and HV strains.

6.1.2. Detection of “non-*aureus*” *Staphylococcus* sp.

The available literature describes *Staphylococcus aureus* as the pathogen of rabbit staphylococcosis. Our results suggest that other species belonging to the *Staphylococcus* genus might have a notable role in the etiology of rabbit staphylococcosis.

6.1.3. Statistical analysis

The association plot and the joint plot figures based on the correspondence analysis of organotropism data revealed some interesting relations. A separation of HV strains from other categories, and a similar behaviour of LV and “Staph sp.” groups has been illustrated. A strong association of HV strains with septicaemia, and LV or “Staph. sp.” strains with cutaneous lesions was also observed.

6.2. Comparative analysis of draft genome sequences

Our results revealed that HV and aHV genotypes are two distinctively different, far-related strains of *Staphylococcus aureus*. Both variants seem to be specific for the domesticated rabbit, but differences between the two variants regarding colonization potential or pathogenicity could only be determined with infection model studies.

The two genomes had several major insertions/deletions, including an approximately 39 kbp phage insertion site in the HV strain and a different 42 kbp phage insertion in another locus in the aHV genome. The two phage insertion sites were somewhat similarly structured regarding major phage component genes, but pairwise nucleotide identity was only 51.8% between these regions. Numerous insertion/deletion sites with length up to 15 kbps are scattered through the two genomes.

If other rabbit originated *selm* sequences have the two mismatches we found, the primer used in virulence determination PCR for rabbit samples should be modified for better amplification results.

The most pronounced heterogeneity was found comparing staphylocoagulase (*coa*) genes, 63.9% pairwise identity on the level of nucleotides, and a 71.6% identity when comparing the translation of these genes (Fig. 3.). We used these two sequences with other 25 staphylocoagulase sequences derived from NCBI Genbank to demonstrate the patristic distance between the two rabbit originated variants (Fig. 4). We observed significant differences comparing the coding sequences for fibrinogen binding factors (*FnbA*, *FnbB*, *ClfA*). Collagen binding protein (*cna*), which was described as a major adhesive factor was not found in any of the rabbit-originated sequences, according to previous studies (Vancraeynest et al. 2004, 2006).

The HV strains found in Hungary should be compared with the strains originating from West-European rabbit farms. Also, a genome sequencing of the aHV strains detected decades ago in Belgium would be important to gain more information on rabbit pathogenic *Staphylococcus aureus* strains.

Controlling infectious diseases is the most important task in industrial animal farming. The pathogens that are able to evade the advanced hygiene practices and medication programmes used standardly in modern agriculture needs to be examined with state-of-the art methodology in order to reveal new possibilities in diagnostics and medicine. The draft whole genome sequences we produced and compared enabled us to identify two nucleotides, where the molecular method used to differentiate these variants might need slight refinement. Synthetic subunit vaccines should be produced in a way, that the immunity would be protective against the most variants of the pathogen. Our results showed that genes encoding extracellular enzymes and toxins are the most conservative sequences, so a subunit vaccine produced from these proteins could be expected to be possibly effective against both variants.

6.3. Genomic analysis of Staphylococcus strains

Staphylococcosis is one of the most important infectious diseases challenging rabbit farmers and veterinarians in modern commercial rabbit meat production. Virulent genotypes can cause epidemics that call for radical solutions, so genotyping the pathogen is a crucial element of diagnostics. Our earlier study showed that a previously rarely isolated atypical variant is the most common among the isolates we collected from Hungarian rabbit farms. The multiplex PCR method used to differentiate highly virulent strains was only capable to indicate that two HV specific sequences were not amplifiable from aHV strains. Analysis of the whole genome showed that HV, aHV and the majority of LV strains form separated genetic clusters with high similarity of strains within each cluster, indicating the clonal origin of the genotypes described 10-15 years ago.

The discriminatory power of the wgMLST method is outstanding: it examines 3897 genes of the pathogen. Some strains in this survey showed genetic polymorphism on more than 3500 loci compared to the root sequence, and even within the same MLST group, hundreds of loci showed polymorphism, which can facilitate very fine differentiation. However, the multiplex PCR method published in 2007 is reinforced by our results, since it can clearly differentiate high and low virulent isolates.

All HV strains formed a closely related cluster and Hungarian strains were almost identical. Staphylococcus aureus ST121 is a globally disseminated hypervirulent clone, important in human medicine on all populated continents (Rao et al., 2015). It is now clearly demonstrated that the infections caused by strains labelled as HV in rabbit-focused diagnostics belong to an epidemic spreading worldwide. The Spanish and Italian strains were isolated from commercial producing units. Most European rabbit meat producers choose from a few hybrid breeds of rabbits, so it is not surprising that virulent pathogens can easily spread around the continent. Most of our HV isolates originated from farms, that are integrated in the production line of a rabbit meat company. The integrator has been providing breeding animals for the farms and most of them rely exclusively on this source of animals. One Hungarian isolate (773-1-10, Ócsa) is separated within the cluster and the production unit of its origin had no contact with the aforementioned integration, thus having an independent supply for genetics. This indicated, that HV strains found multiple ways to contaminate commercial rabbit production in Hungary.

The wgMLST also confirmed, that aHV genotypes are almost as closely related, as strains of the HV cluster. It is well known that in closed, industrial rabbit populations, different Staphylococcus variants can cause health problems. This genotype was detected

in 1994 on four Belgian farms, where the rabbits originated from the Czech Republic. The solution for the epidemic was the eradication of the affected populations, and this genotype was not reported until a Hungarian survey in 2014. Our results clarified that these isolates, until now only identified by amplification of a few selected genetic elements, constitute a group of closely related, most probably clonal specimens of *Staphylococcus aureus*. The aHV group has a novel MLST pattern. Beside the unique gmk and pta sequences, other ST alleles were also very rare variants. At the time of our study, 5993 S. aureus MLST profiles are listed in the PubMLST database, and among them only 4 and 3 contain the same allelic variant of arc and glpF, respectively (Jolley et al., 2018).

The whole genome of the LV strains also provided interesting new discoveries. The majority of the strains formed a cluster of very closely related strains, despite that both MLST and spa sequence typing methods resulted in great diversity. The 7 genes of MLST and the single spa sequence would have distributed these 10 strains into 7 different clusters, while analysis of 3897 loci revealed that within the main cluster only an average of 1,3% of the genetic features was different. Based on the wgMLST, the clonal origin of this cluster also seems very probable.

The second branch within the LV genotype only contained 2 strains, isolated in the same diagnostic case from two different animals. This rabbitry is a small-scale rural unit, where technological problems and poor hygiene could have contributed to the infection of the animals. ST1 is an important variant of this pathogen in both human and veterinary medicine. The clonal lineage has a low host-specificity, thus the presence of such strains may imply human originated contamination or poses a risk for zoonotic transmission of the bacteria.

Attili et al. examined the genotype of 96 S. aureus strains, all isolated at the same farm, but from both animals and farm workers. All of the strains were classified as LV, but similarly diverse with spa typing. 5 different spa types (t094, t491, t605, t2036, t2802) were detected, none of them were similar to the spa type of the strains from Hungary (Attili et al., 2020).

Sequencing Staphylococcus strains, which did not contain the femA gene, thus classified as Staphylococcus sp. before, held the promise of identifying new pathogenic species related to rabbit staphylococcosis. However, the results showed that these strains are closely related to bacteria, which are members of the dermal microbiota, but are also known for their possible etiological role in dermatitis (Argemi et al., 2019). All of these strains were isolated from contaminated skin lesions, their presence might be secondary to physical trauma of the dermis, but also could be the origin of the disease. Animal

experimental models (Hermans et al., 2000a; Meulemans et al., 2011) could decide this question, however nor the negligible prevalence, nor the clinical importance of this infection justify such research.

7. Conclusions

In rabbits, *Staphylococcus aureus* bacteria infect small dermal lesions and invade subcutaneous tissue (Okerman et al., 1984). The infections have similar clinical appearance, lesions of pododermatitis, subcutaneous abscesses and mastitis caused by this agent. Internal organs can also be evaded, and abscesses can observe in lungs, uterus and liver. In the flock, level high virulence *S. aureus* strains leads to chronic problems and epidemic spread of the disease.

Despite extensive research on the pathogen and the disease, current medicine fails to eradicate rabbit staphylococcosis. Results from this study indicate that atypical highly virulent strains are the most important in Hungarian farms, but typical HV strains are also present sporadically. The origin and dissemination of both typical and atypical virulent types would call for further investigations. The frequency and pattern of HV strain isolation raises questions about its epidemiology and a possible interaction of typical and atypical highly virulent strains. Further studies are needed to address this hypothesis.

Virulent genotypes can cause epidemics that call for radical solutions, so genotyping the pathogen is a crucial element of diagnostics. Our earlier study showed, that a previously rarely isolated atypical variant is the most common between the isolates we collected from Hungarian rabbit farms. The multiplex PCR method was only capable to indicate that two HV specific sequences are not amplifiable from aHV strains.

If other rabbit originated *selm* sequences have the two mismatches we found, the primer used in virulence determination PCR for rabbit samples should be modified for better amplification results.

The HV strains found in Hungary should be compared with the strains originating from West-European rabbit farms. A genome sequencing of the aHV strains detected decades ago in Belgium would be important to gain more information on rabbit pathogenic *Staphylococcus aureus* strains.

Our present results revealed that HV and aHV genotypes are two distinctively different, far-related strains of *Staphylococcus aureus*. Both variants seem to be specific for the domesticated rabbit, but differences between the two variants regarding colonization potential or pathogenicity could only be determined with infection model studies.

Next generation sequencing is clearly the future way of epidemiology. WGS in this project was conducted at a lower expense per sample, than the 8 PCR reactions and sequencing needed for conventional MLST and spa typing cost. The PCR and Sanger sequencing methods remain useful for daily diagnostics, but their limitations are clearly demonstrated with the LV strains in this study, where very similar genomes had minor differences on the loci where the conventional method focuses.

Our results confirmed that several different genotypes of Staphylococcus aureus can cause clinical problems in commercial rabbit farms. The clonal origin of HV strains was confirmed decades ago (D. Vancraeynest et al., 2006) and the significant pathogenicity of this genotype was experimentally proven (Hermans et al., 2000a; Meulemans et al., 2011). The most prevalent genotype in Hungarian rabbit farms is aHV strains, which have a similar clonal origin, but very distantly related to HV strains. LV strains are considered as less pathogenic variants based on biochemical properties related to human- or poultry-associated strains. The minor cluster fits into this idea, but the major cluster suggests that these strains might represent a third rabbit-associated clonal lineage. The similarity between the three main clusters is very low, around 22.8% of all loci have the same allele between the aHV and LV strains, and only 6.4% between the HV-aHV or HV-LV genotypes. These strains can coexist within the same farm, but they have never been isolated simultaneously from the same animal. Microbes of the skin and mucosal microbiota compete with each other for space and resources and the interference between the different variants of the same pathogenic species would be an interesting topic for future research. Some variants could be less virulent but more successful in colonisation, such strains could be useful to decrease the spread of more aggressive variants.

8. Overview of new scientific results

1. Detailed molecular characterization of clinical Staphylococcus isolates originating from Hungary. Implementation of a polymerase chain reaction method in clinical diagnostic services. Identification os three different virulence variants amongst Hungarian isolates. Presence or interaction of multiple variants within one population was never examined or reported before.
2. The statistical analysis on data about genotype and organ of origin of the isolates revealed that different genotypes have significantly different organotropism. HV strains are highly correlated to septicaemia. LV and non-aureus *Staphylococcus* strains are much more likely to only cause local lesions on the skin.
3. Publication of the first draft of the whole genome sequence of a rabbit HV *Staphylococcus aureus* strain, 2.684.832 nucleotides were assembeled, 2395 annotated genes, 50 tRNAs and 5 rRNAs were obtained. The first reported draft of the whole genome of an aHV strain was also our product, 2.631.087 nucleotides, 2567 annotated genes, 52 tRNAs and 11 rRNAs were reported in our study.
4. We compared the two draft genomes in two different approaches. Pairwise alignment of the two drafts revealed that the two virulent variants are in fact two very different, far related variants of *Staphylococcus aureus*. Comparing 63 virulence-related genes we concluded that EC enzymes of the two genotypes are very similar, but the whole enterotoxin gene cluster is absent from the aHV strain. This kind of virulence factor gives a remarkable advantage to a microorganism, and must have a fundamental role in the pathogenicity of HV strains. Surface components and adhesion factors were proven to be notably different between HV and aHV strains. This result details the illustration of the evolutionary distance between the two variants, and can explain the differences in organotropism concluded earlier.
5. Whole-genome sequencing of 50 Stapylococcus isolates, assembly of draft genome sequences, publicating all data to open digital repositories.
6. Epidemiologic analysis of 64 rabbit-originated Staphylococcus isolates representing 4 European countries with whole-genome MLST. Reinforcment of clonal origin and international dissemination of HV strains, demonstration of two new clonal clustrers (aHV-ST5993, LV-ST2855) of the pathogen.

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10. Scientific publications

10.1. Publications involved in the thesis

Német, Zoltán ; Szenci, Ottó ; Biksi, Imre

A házinyúl staphylococcosis**a : Irodalmi áttekintés és hazai tapasztalatok: Irodalmi áttekintés és hazai tapasztalatok** Staphylococcosis **in domestic rabbit. Literature review and domestic experiences**

MAGYAR ÁLLATORVOSOK LAPJA 133 pp. 720-726. , 7 p. (2011)

Német, Zoltán ; Albert, Ervin ; Nagy, Krisztina ; Csuka, Edit ; Dán, Ádám ; Szenci, Ottó ; Hermans, Katleen ; Balka, Gyula ; Biksi, Imre

Virulence type and tissue tropism of *Staphylococcus* strains originating from Hungarian rabbit farms

VETERINARY MICROBIOLOGY 193 pp. 1-6. , 6 p. (2016)

Nemet, Z ; Albert, E ; Nagy, T ; Olasz, F ; Barta, E ; Kiss, J ; Dan, A ; Banyai, K ; Hermans, K ; Biksi, I

Draft Genome Sequence of an Atypical Highly Virulent Rabbit *Staphylococcus aureus* Strain.

GENOME ANNOUNCEMENTS 5 : 42 Paper: e01049-17 (2017)

Nemet, Z ; Albert, E ; Nagy, T ; Olasz, F ; Barta, E ; Kiss, J ; Dan, A ; Banyai, K ; Hermans, K ; Biksi, I

Draft Genome Sequence of a Highly Virulent Rabbit *Staphylococcus aureus* Strain.

GENOME ANNOUNCEMENTS 3 : 4 Paper: e00461-15 , 2 p. (2015)

10.2. Other publications

Magyar, Tibor ; Ujvári, Barbara ; Szeredi, Levente ; Virsinger, Norbert ; Albert, Ervin ; Német, Zoltán ; Csuka, Edit ; Biksi, Imre

Re-emergence of bovine haemorrhagic septicaemia in Hungary

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Dip, R ; Nemet, Z ; Schiessl, B ; Klein, U ; Strehlau, G

Efficacy and tolerability of early administration of valnemulin hydrochloride premix on epizootic rabbit enteropathy

VETERINARY JOURNAL 204 : 3 pp. 309-314. , 6 p. (2015)

Sándor, Hornok ; Anita, Mester ; Nóra, Takács ; Ferenc, Baska ; Gábor, Majoros ; Éva, Fok ; Imre, Biksi ; Zoltán, Német ; Ákos, Hornyák ; Szilárd, Jánosi et al.

Sarcocystis-infection of cattle in Hungary

PARASITES AND VECTORS 8 Paper: 69 , 6 p. (2015)

Takács, Norbert ; Albert, Ervin ; Kiss, Krisztián ; Német, Zoltán ; Biksi, Imre

Sertések légzőszervi megbetegedéseinek elkülönítő kórjelzése I. Választás előtti kórképek: Differential diagnosis of porcine respiratory diseases I

MAGYAR ÁLLATORVOSOK LAPJA 137 : 1 pp. 15-24. , 10 p. (2015)

Vincze, B ; Szelényi, Z ; Horváth, A ; László, N ; Németh, Z ; Szenci, O

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A sercegő üszök (Gangraena emphysematosa) előfordulása egy hazai tejelő tehenészetben. Esetleírás: Incidence of black leg disease (gangrenous myositis) in a Hungarian dairy cow herd

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A bal oldali oltógyomor-helyzetváltozás előfordulásával és diagnosztizálásával kapcsolatos hazai tapasztalatok tejelő tehenészetekben: Experiences about the incidence and diagnosis of left-sided abomasum displacement in Hungarian dairy herds

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Outbreak of Klebsiella oxytoca enterocolitis on a rabbit farm in Hungary

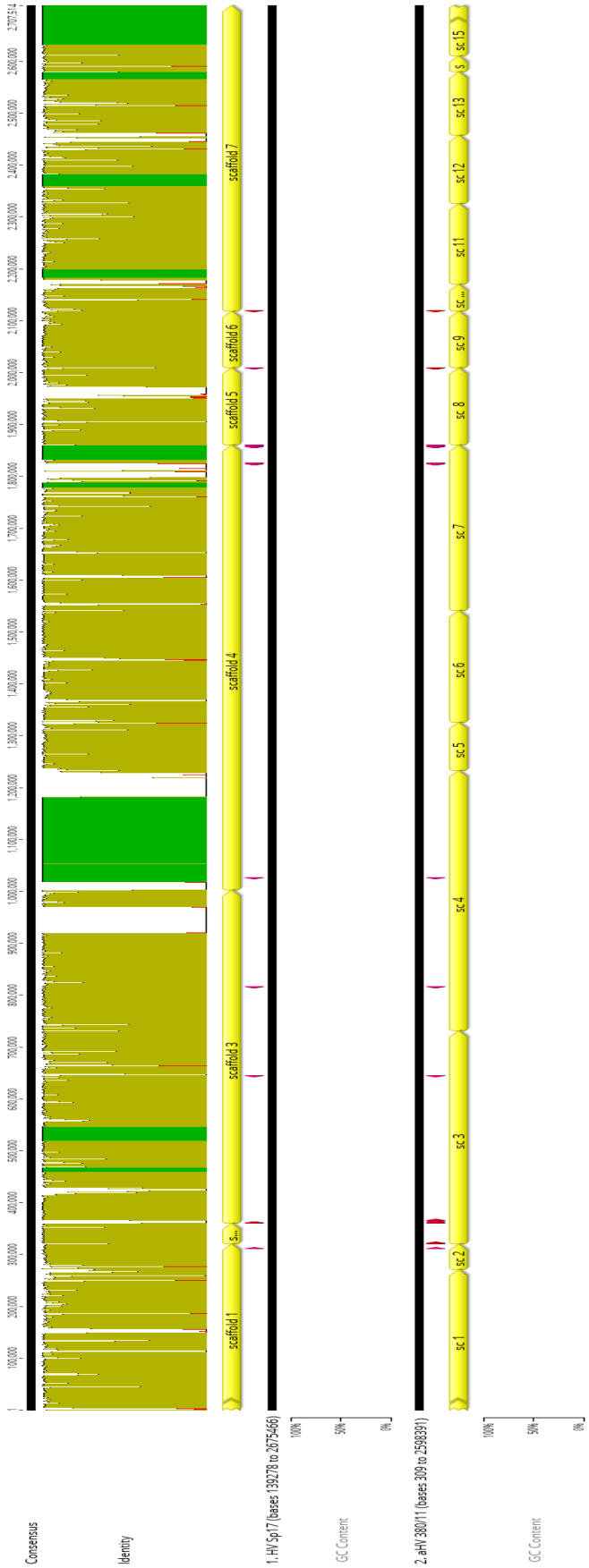
VETERINARY RECORD 168 : 9 pp. 243-243. , 1 p. (2011)

Német, Zoltán ; Biksi, Imre ; Szenci, Ottó ; Kis, Tamás ; Csere, István

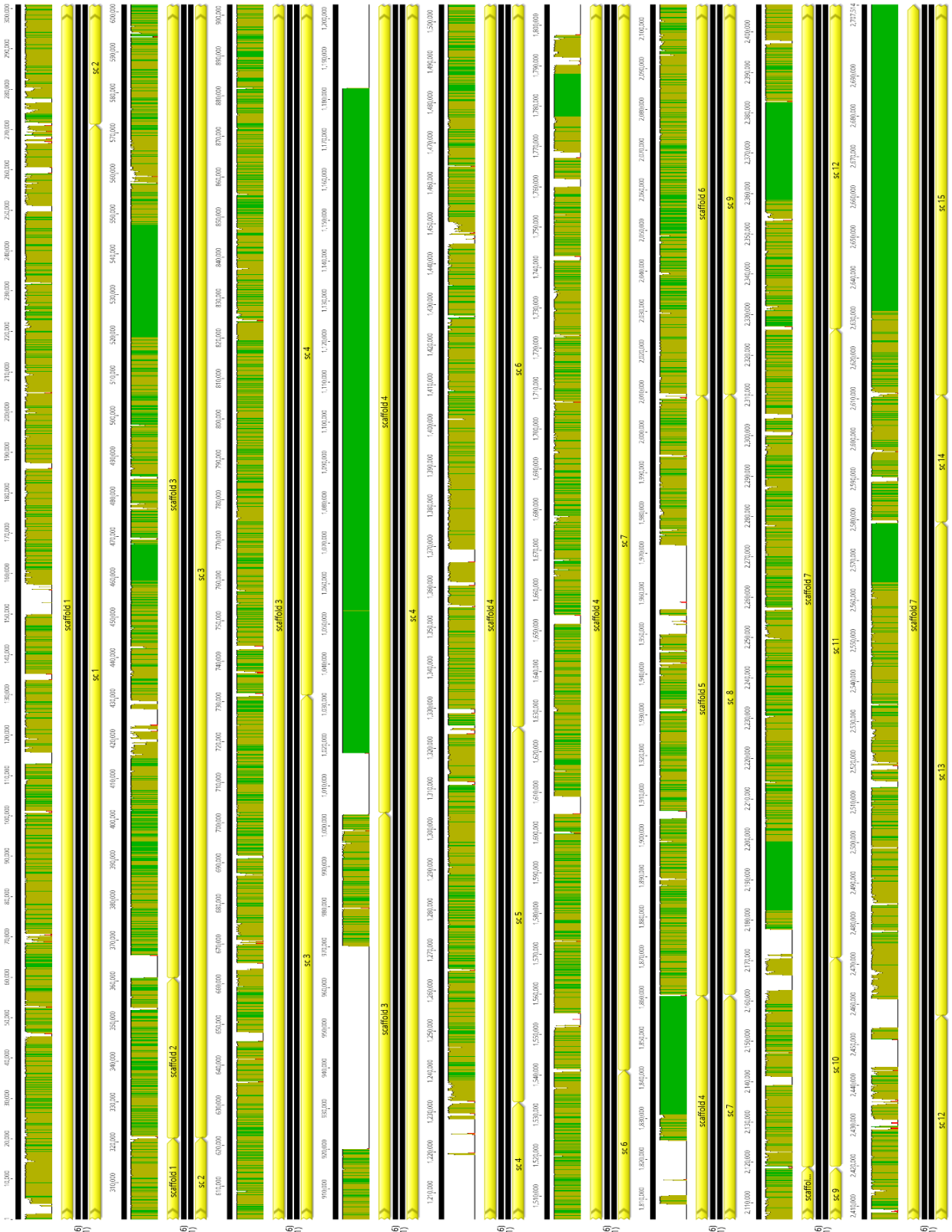
A nyulak járványos enteropathiája okozta veszteségek megelőzése bacitracin- és oxitetraciklintartalmú készítménnyel hazai hízónyúlállományokban

MAGYAR ÁLLATORVOSOK LAPJA 132 : 6 pp. 361-366. , 6 p. (2010)

11. Appendix



9. Figure: ***General overview of the alignment of the two draft genomes****. Scaffold order and orientation was determined arbitrary to facilitate visualisation.*



A1 Figure: ***Detailed alignment of the two genomes***

Table A1: Major virulence factor coding genes in Staphylococcus aureus, comparison of sequences found in the examines HV and aHV strain.

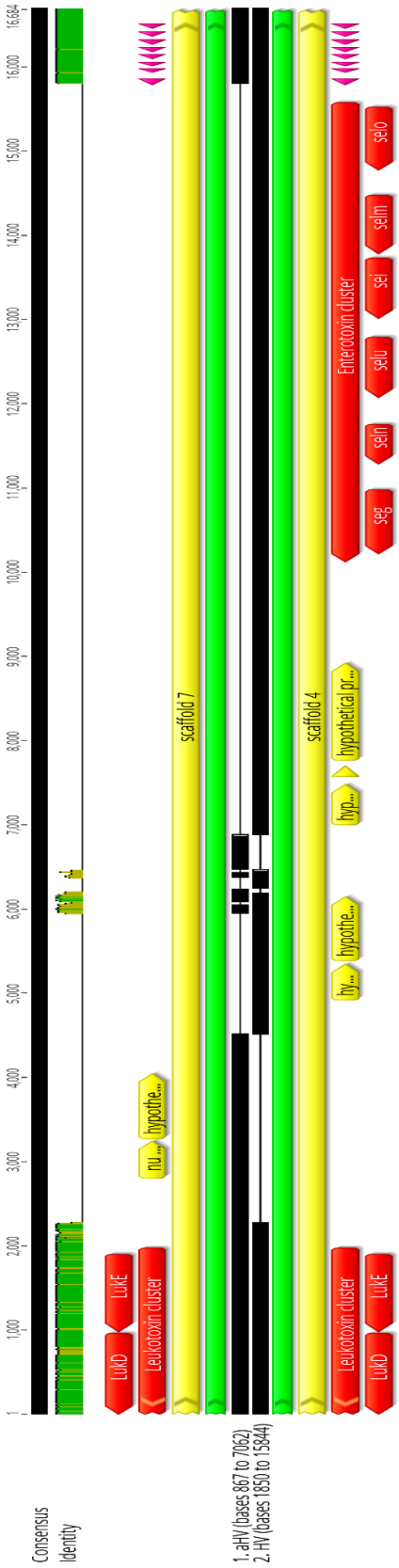
MSCRAMMs (Microbial surface components recognizing adhesive matrix molecules)		**HV (Sp17)**		**aHV (380/11)**		
Name of virulence factor	**Function**	**Gene abbreviation**	**Length (bp)**	**Gene at parallel locus**	**Length (bp)**	**Identity %**
Fibronectin-binding protein A and B	Fibronectin, fibrinogen, ECM binding	FnbA	3012	FnbA	2988	86,1
Fibronectin-binding protein A and B	Fibronectin, fibrinogen, ECM binding	FnbB	2793	FnbB	2754	87,4
Clumping factor A	Binds to fibrinogen	ClfA	2982	ClfA	2772	76,5
Clumping factor B	Binds to fibrinogen	ClfB	2448	Partial gene at scaffold end		
Elastin binding protein	Binds elastin	EbpS	1455	EbpS	1461	95,2
Collagen binding protein	Binds collagen	cna	-	cna	-	
Serin-aspartic acid-rich protein	ECM binding	SdrC	2799	SdrC	2826	89,3
Serin-aspartic acid-rich protein	ECM binding	SdrE	3990	SdrC-like unknown adhesin	3894	73,8
S. aureus surface protein	ECM binding	SasA	6450	SasA	6450	100
S. aureus surface protein	ECM binding	SasC	6561	SasC	6564	95,1
S. aureus surface protein	ECM binding	SasC	7437	SasC	7439	95,9
S. aureus surface protein	ECM binding	SasG	591	IN/DEL		
S. aureus protein A	Binds immunoglobin Fc portions, von Willebrand factor, TNFR1, eukaryotic gC1q-R	spa	1527	spa	1527	94,8
S. aureus protein A	Binds immunoglobin Fc portions, von Willebrand factor, TNFR1, eukaryotic gC1q-R	hypothetical protein similar to spa	483	hypothetical protein similar to spa	471	87
Polysaccharide intercellular adhesin (PIA)	Biosynthesis of N-acetylglucosaminilltransferase, capsular polysaccharid expression	icaA	1113	icaA	1113	100
Polysaccharide intercellular adhesin (PIA)	Biosynthesis of N-acetylglucosaminilltransferase, capsular polysaccharid expression	icaB	873	icaB	873	100
Polysaccharide intercellular adhesin (PIA)	Biosynthesis of N-acetylglucosaminilltransferase, capsular polysaccharid expression	icaC	1053	icaC	1053	100
Polysaccharide intercellular adhesin (PIA)	Biosynthesis of N-acetylglucosaminilltransferase, capsular polysaccharid expression	icaD	306	icaD	306	100

Table A1. continued

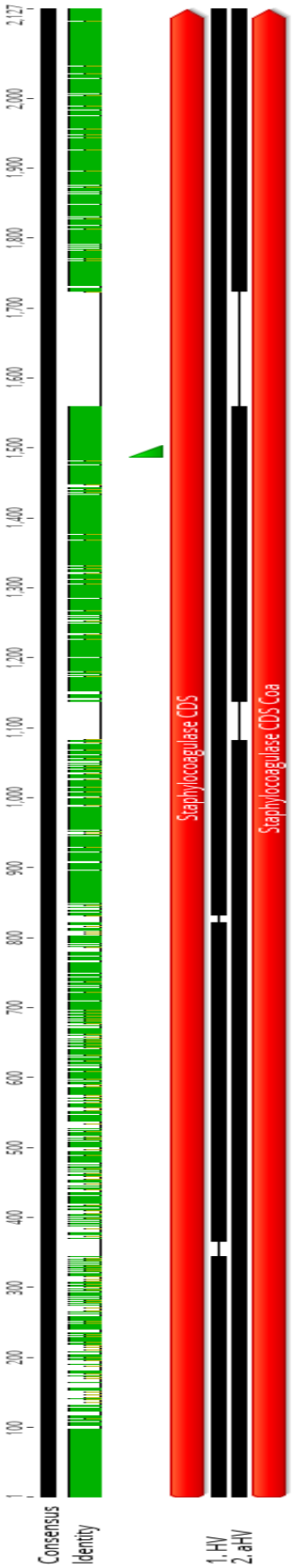
Extracellular enzymes		HV (Sp17)		aHV (380/11)		
Name of virulence factor	**Function**	**Gene abbreviation**	**Length (bp)**	**Gene at parallel locus**	**Length (bp)**	**Identity %**
Catalase	Inactivates free hydrogen peroxide	Catalase	1518	Catalase	1518	99,4
Alkylhydroperoxide reductase	Residual catalase activity	Alkylhydroperoxide reductase	570	Alkylhydroperoxide reductase	570	99,5
Alkylhydroperoxide reductase	Residual catalase activity	Alkylhydroperoxide reductase	1524	Alkylhydroperoxide reductase	1524	98,3
Thioredoxin and thioredoxin reductase	Inactivates ROS	Thioredoxin	321	Thioredoxin	321	99,1
Thioredoxin and thioredoxin reductase	Inactivates ROS	Thioredoxin	297	Thioredoxin	297	99
Thioredoxin and thioredoxin reductase	Inactivates ROS	Thioredoxin	315	Thioredoxin	315	99
Thioredoxin and thioredoxin reductase	Inactivates ROS	Thioredoxin	312	Thioredoxin	312	99,7
Thioredoxin and thioredoxin reductase	Inactivates ROS	Thioredoxin	564	Thioredoxin	564	100
Thioredoxin and thioredoxin reductase	Inactivates ROS	Thioredoxin	345	Thioredoxin	342	99,7
Thioredoxin and thioredoxin reductase	Inactivates ROS	Thioredoxin reductase	936	Thioredoxin reductase	936	99,4
Thioredoxin and thioredoxin reductase	Inactivates ROS	Thioredoxin reductase	987	Thioredoxin reductase	987	99,1
Thioredoxin and thioredoxin reductase	Inactivates ROS	Thioredoxin reductase	1035	Thioredoxin reductase	1035	98,9
Glycerol ester hydrolases	Degrade triacylglycerols	lip	2046	lip	2046	100
Glycerol ester hydrolases	Degrade triacylglycerols	geh	1056	geh	1056	98
Glycerol ester hydrolases	Degrade triacylglycerols	geh	429	geh	480	96,3
Fatty acid-modifying enzyme	Modifies fatty acids	esterase/lipase	735	esterase/lipase	735	98,6
Fatty acid-modifying enzyme	Modifies fatty acids	esterase/lipase	969	esterase/lipase	969	98,9
Proteases	Complex network of zymogens with pleiotropic effects on pathogenesis	Staphopain (sspA)	1167	Staphopain (sspA)	1167	99,1
Proteases	Complex network of zymogens with pleiotropic effects on pathogenesis	aureolysin (Aur)	1530	aureolysin (Aur)	1530	99,4
O-acetyltransferase	Peptidoglycan O-acetylation	OatA	1815	OatA	1812	98,8
Staphylokinase	Plasminogen activator	Sak	627	Sak	627	96,7
Enolase	Catalyzes phosphor-glycerate to phosphoenol-pyruvate; binds to laminin, also MSCRAMM	Enolase, eno	1305	Enolase, eno	1305	99,7

Table A1. continued

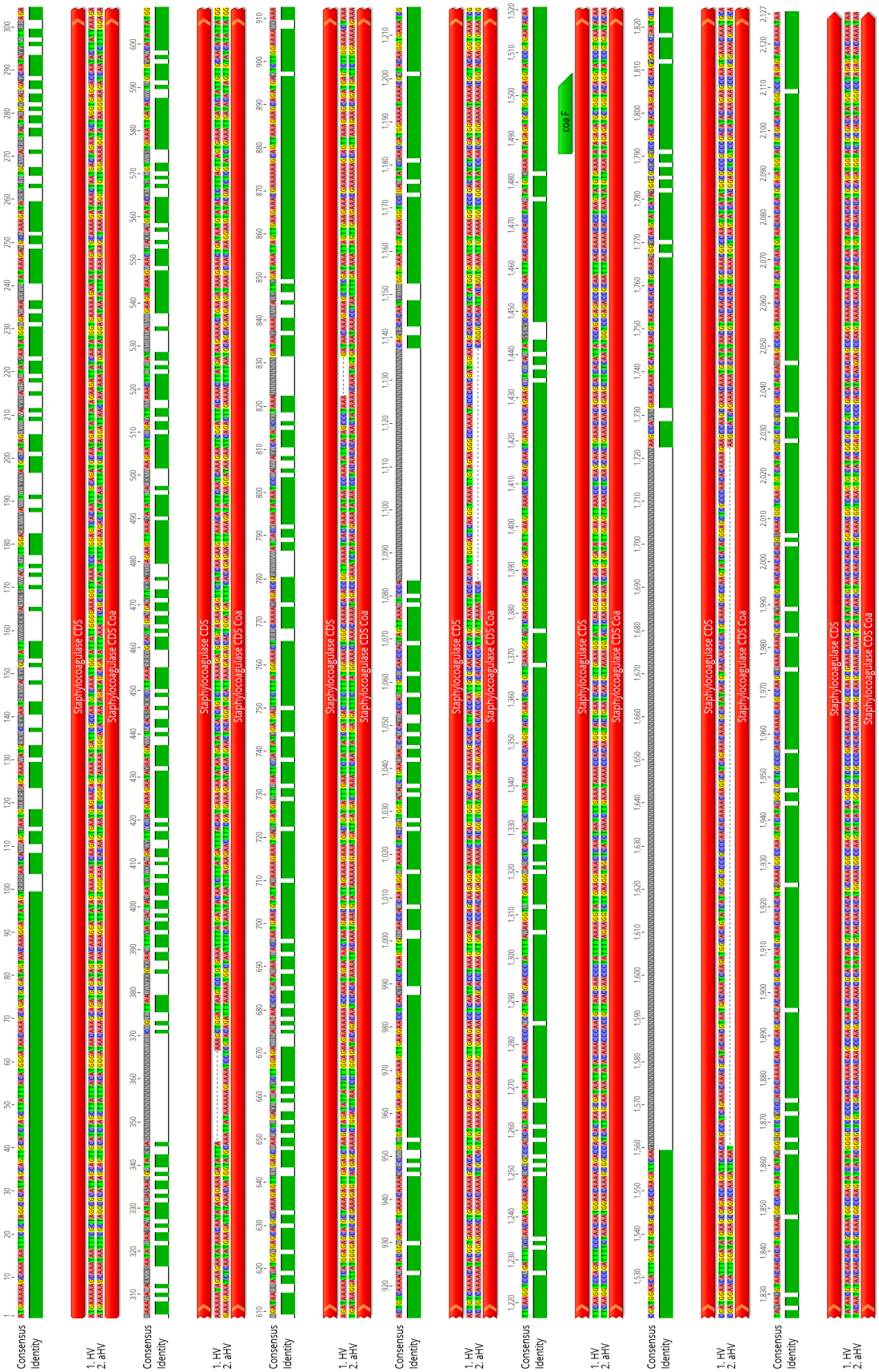
SERAMs (Secreted expanded repertoire adhesive molecules)		HV (Sp17)		aHV (380/11)		
Name of virulence factor	**Function**	**Gene abbreviation**	**Length (bp)**	**Gene at parallel locus**	**Length (bp)**	**Identity %**
Extracellular adherence protein	Binds to ECM molecules; binds to ICAM-1; blocks the LFA-1-ICAM-1 interaction	Eap/Map	1437	Eap/Map	1749	79,3
Extracellular adherence protein	Binds to ECM molecules; binds to ICAM-1; blocks the LFA-1-ICAM-1 interaction	Eap/Map	291	Eap/Map	291	97,6
Coagulase	Activates prothrombin	Coa	2097	Coa	1911	57,9
ECM binding protein	Interacts with ECM	Emp + ssp	1020	Emp + ssp	1023	96,3
Extracellular fibrinogen binding protein	Binds to fibrinogen; binds to complement factors C3b and C3d	Efb	498	Efb	498	98,4
Extracellular fibrinogen binding protein	Binds to fibrinogen; binds to complement factors C3b and C3d	hypothetical protein similar to Efb	318	hypothetical protein similar to Efb	330	95,5
Sbi	Binds to IgG	SBI	1311	SBI	1311	100
Toxins						
α-toxin	Pore formation in erythrocytes and monocytes	α-toxin	936	α-toxin	960	99
β-Hemolysin (sphingomyelinase C)	Lysis of cytokine-containing cells	β-Hemolysin	996	β-Hemolysin	996	99
δ-hemolysin	Neutrophil and monocyte binding	δ-hemolysin	136	δ-hemolysin	135	97,8
Bicomponent toxins: g-hemolysin; Panton-Valentine leukocidin; leukocidins D, E, M	g-toxin is hemolytic PVL stimulates and lyses neutrophils and macrophages	hlgA	966	hlgA	966	100
Bicomponent toxins: g-hemolysin; Panton-Valentine leukocidin; leukocidins D, E, M	g-toxin is hemolytic PVL stimulates and lyses neutrophils and macrophages	hlgB	978	hlgB	978	100
Bicomponent toxins: g-hemolysin; Panton-Valentine leukocidin; leukocidins D, E, M	g-toxin is hemolytic PVL stimulates and lyses neutrophils and macrophages	hlgC	948	hlgC	948	100
Bicomponent toxins: g-hemolysin; Panton-Valentine leukocidin; leukocidins D, E, M	g-toxin is hemolytic PVL stimulates and lyses neutrophils and macrophages	LukD	983	LukD	984	97,9
Bicomponent toxins: g-hemolysin; Panton-Valentine leukocidin; leukocidins D, E, M	g-toxin is hemolytic PVL stimulates and lyses neutrophils and macrophages	LukE	936	LukE	936	98,6
Bicomponent toxins: g-hemolysin; Panton-Valentine leukocidin; leukocidins D, E, M	g-toxin is hemolytic PVL stimulates and lyses neutrophils and macrophages	LukF	1017	LukF	1017	97,1
Bicomponent toxins: g-hemolysin; Panton-Valentine leukocidin; leukocidins D, E, M	g-toxin is hemolytic PVL stimulates and lyses neutrophils and macrophages	LukS	951	LukS	1056	99,1
Toxins with superantigen activity (TSS)	Food poisoning; confer TSS	seg	777	IN/DEL		
Toxins with superantigen activity (TSS)	Food poisoning; confer TSS	sei	732	IN/DEL		
Toxins with superantigen activity (TSS)	Food poisoning; confer TSS	seln	498	IN/DEL		
Toxins with superantigen activity (TSS)	Food poisoning; confer TSS	selm	720	IN/DEL		
Toxins with superantigen activity (, TSS)	Food poisoning; confer TSS	selo	765	IN/DEL		
Toxins with superantigen activity (TSS)	Food poisoning; confer TSS	selu	732	IN/DEL		
Anti-inflammatory peptides						
FPR-like 1 inhibitory protein	Binds to FPR-like 1 receptor	flr	399	flr	402	81,4



10. Figure: ***Enterotoxin gene cluster in the HV strain****. The aHV genome did not have these features. In routine diagnostics the amplification of selm is only possible with HV strains.*



11. Figure: ***Identity between the two staphycoagulase (coa) sequences.*** *This gene is the least similar amongst the virulence genes examined.*



A2. Figure: ***Pairwise alignment of the two staphycoagulase (coa) sequences, based on the nucleotide sequences.*** *This gene is the least similar amongst the virulence genes examined.*

12. Acknowledgements

First I have to express my gratitude to my supervisor, Imre Biksi for his continuous support and endless patience during this long time we have been working together. It has never been easy, but so it goes. My closest collegues, Ervin Albert, Edit Csuka, Ádám Dán and Zoltán Pápai deserve the same respect and honour, without them this work would not have been possible at all.

The work started as diagnostic services and sample collection for scientific purposes ended up as a second occupation as a veterinary consultant at the rabbit producing integration of Tetrabbit Ltd. Above the fruitful professional, scientific and business relationship I feel I became part of a large, diverse, but very familiar community. For this I have to express my greatest appreciation to Ferenc Sándor, Andrea Kiss and their whole family, Johann and Zsuzsanna Gerber and Attila Szalay. A special thank has to be granted for the best collegues, Mónika Mohaupt and Csongor Demeter, who never hid or envied their hard-earned special experience, which proved to be indispensable for my research.

A friend and collegue, who is always there at the time of need, but never afraid to say what is true, Gyula Balka, has to be acknowledged at the highest levels for his honest and critical approach for everything I did.

My work is strongly based on molecular genetics and bioinformatics, which are not common skills amongst veterinary practicioners. It is my honor to acknowledge Endre Barta, Tibor Nagy and Ferenc Olasz from the Agricultural Biotechnology Centre, who were kind enough to sacrifice a significant amount of their precious time for my project. Rita Sipos and Biomi Ltd. also provided essential services and magnanimous help with the sequencing project.

I can never be grateful enough to my high school teachers, who introduced me into life sciences, Bernát Baumgartner, Attila Drozdík and József Rékási.

Last but not least, I am grateful to my wife and family, who always supported and encouraged me to keep going on.