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Short communication

CRISPR tracking reveals global spreading of antimicrobial resistance genes by *Staphylococcus* of canine origin

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ABSTRACT

The close contact between pets and their owners is a potential source for microorganisms and genetic material exchange. *Staphylococcus* species considered as harmless inhabitants of animals' and humans' microbiota can act as reservoirs of antimicrobial resistance genes to more virulent species, thereby increasing their potential to resist drug therapy. This process could be inhibited by the antiplasmid immunity conferred by CRISPR systems. On the other hand, CRISPR spacer sequences can be explored as molecular clocks to track the history of genetic invasion suffered by a bacterial strain. To understand better the role of domestic dogs in human health as an antimicrobial resistance genes source, we analyzed 129 genomes of *Staphylococcus* strains of canine origin for the presence of CRISPR systems. Only 8% of the strains were positive for CRISPR, which is consistent with *Staphylococcus* role as gene reservoirs. The plasmidial origin or some spacers confirms the unsuccessful attempt of plasmid exchange in strains carrying CRISPRs. Some of these systems are within a staphylococcal cassette chromosome *mec* (SCC*mec*), sharing 98% of identity between their harboring strains. These CRISPRs' spacers reveal that this SCC*mec* was transferred between canine *S. pseudintermedius* strains, then to *S. schleiferi* and to *Staphylococcus* strains isolated from human beings. Our findings shows genetic evidence for the global spreading of pathogenic bacteria and the antimicrobial resistance genes carried by them and reinforce that, in the age of antimicrobial resistance, it is imperative that drug therapies consider the integrated nature of the relationship between pets and humans.

1. Introduction

The frequent and close contact between human beings and their pets is a central source for exchange of microorganisms, a process that can significantly shape the microbial communities of both counterparts (Song et al., 2013). Bacteria of the *Staphylococcus* genus are natural inhabitants of both humans and animals' microbiota and, even though most species are harmless residents, some are responsible for causing primary or opportunistic infections, such as *S. aureus* and *S. pseudintermedius*, the latter being a major canine pathogen (Becker et al., 2014; Rossi et al., 2018).

In addition, staphylococcal species considered as inoffensive can pose a threat to their hosts' health by acting as reservoirs of antimicrobial resistance genes to more pathogenic strains, which enhance their potential to resist antibiotic therapy (Cafini et al., 2016; Rossi et al., 2017). However, this relative ease with which staphylococcal strains transmit resistance genetic information to each other could be hampered by the presence of CRISPR systems. These systems are estimated to exist in about 50% of bacterial genomes and their antiplasmid activity limits horizontal gene transfer in *Staphylococcus* by targeting DNA (Hatoum-Aslan et al., 2014; Marraffini and Sontheimer, 2008).

CRISPR, the abbreviation for Clustered Regularly Interspaced Short Palindromic Repeats, is an array of short direct identical sequences (direct repeats, DR) separated from each other by other equally short (usually 20–40 bp) sequences called spacers. These spacers are fragments of exogenous DNA or RNA, such as bacteriophages and plasmids, which are incorporated by the CRISPR-associated (Cas) proteins upon an event of invasion, as a part of bacterial immunity mechanism. The Cas proteins further use these spacers in an interference process to keep exogenous DNA or RNA from establishing themselves within the cell (Sorek et al., 2008). Because the spacers are incorporated in a chronological order, CRISPR sequences can be explored as molecular clocks that reveal the history of genetic invasion suffered by the bacteria harboring the system. We have recently shown that from human origin may carry CRISPR systems within mobile genetic elements, with its

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spacers indicating that these elements were transferred between different species of *Staphylococcus*, which may also have involved a strain of canine origin (Rossi et al., 2017). One of these elements is the Staphylococcal Cassette Chromosome *mec*, a type of genomic island that carries the methicillin-resistance gene *mecA* (or the recently described *mecC*), encoding a low-affinity penicillin binding protein, also called PBP2 A. SCC*mec* elements also contain a complex of *ccr* genes, which encode recombinases that are involved in the excision, circularization and insertion of the cassette in the chromosome of staphylococci (Firth et al., 2018).

To better understand the role of domestic dogs in human health as reservoirs of *Staphylococcus* strains and antimicrobial resistance genes, this study aimed to evaluate the presence of CRISPR systems and track their history of transfer among different *Staphylococcus* species and strains of canine origin.

2. Materials and methods

2.1. Genomes analyzed

The genomes of 129 *Staphylococcus* isolates, collected from domestic dogs, were analyzed (Table S1). These sequences were previously publicly available at Genbank, either as complete genomes or assembled in contigs, and belong to four different species: *S. aureus* (n = 53), *S. pseudintermedius* (n = 74), *S. haemolyticus* (n = 1) and *S. cohnii* (n = 1). They were isolated from nine different nations of four continents: Australia, Denmark, France, Hong Kong, Netherlands, Spain, Sri Lanka, United Kingdom and United States of America. All genomes were analyzed for antimicrobial resistance genes with Resfinder (Zankari et al., 2012)

2.2. Analysis of CRISPR presence

All the genomes were analyzed for the presence of CRISPR sequences and Cas proteins with CRISPRCasFinder (Couvin et al., 2018), following the standard parameters of the software. Only complete systems, *i.e.*, those who contained both the CRISPR arrangements and Cas proteins were considered in further analyses.

2.3. Analysis of the genetic context of the CRISPR sequences

The locus or contig of each genome containing the CRISPR sequence had their open reading frames (ORFs) properly annotated with the Artemis software (Carver et al., 2012), and NCBI's BLASTp tool, for the definition of each system's type. The presence of neighbor antimicrobial resistance genes was searched with Resfinder (Zankari et al., 2012), and the presence of insertion sequences that could influence the system's mobility was investigated with ISFinder (Siguier et al., 2012).

2.4. Investigation of the spacer's origins

The spacer sequences of each CRISPR found were manually removed and confronted against each other for redundancy with NCBI's BLASTn tool. Then, a file containing the unique sequences was analyzed again against the Genbank sequence database with BLASTn for investigation of the origins of each spacer.

3. Results

3.1. CRISPR abundance, features and antimicrobial resistance genes

Only 10 out of 129 isolates (8%) were positive for the presence of CRISPR systems (Fig. 1). Based on their arrangements of Cas proteins, they are either of type IIC (containing three *cas* genes, for proteins Cas1–2 and Cas9) or of type IIIA (containing nine *cas* genes, for proteins Cas1–2, Csm2–6, Cas6 and Cas10). All of the CRISPR-positive strains belong to the *S. pseudintermedius* species. Most of them harbor only one CRISPR system, but the strain MRSP1019 has one system of each type, and MRSP424 has two CRISPR systems of type IIIA and one system of type IIC. With exception of one of the systems from MRSP424, all the other type IIIA systems have CRISPR sequences upstream and downstream the *cas* operon, while in type IIC, the sequences are all in the downstream position.

While all the strains studied present antimicrobial resistance genes, they are clearly more abundant among *S. pseudintermedius*, which are mostly multidrug resistant and carry an impressive average of 10.8 ± 3.2 different genes, in contrast to 4.1 ± 1.5 genes harbored by *S. aureus* strains (Table S2). However, there is no clear statistically significant ($p < 0.05$ in Student's *t* test) relationship in the number of resistance genes in the CRISPR-lacking and CRISPR-carrying strains.

3.2. Origins of spacers

The 13 CRISPRs described in here have from three to 23 spacers (Fig. 1). Together they make up to 202 sequences, 125 of which are unique, as many spacers are found in the CRISPR locus of at least two different strains (Table S3). From those 125 spacers, the origin of only 28 (22%) have known counterparts in the public sequences database of Genbank, 24 (19%) of which come from bacteriophages and 4 (3%) come from plasmids previously described in other *Staphylococcus* strains (Table S3). These plasmids come from different staphylococcal species and the spacers target either hypothetical proteins or intergenic regions (Table S4).

3.3. Some type IIIA CRISPR systems are located within SCCmec elements

At least three type IIIA CRISPR systems, those belonging to *S. pseudintermedius* MRSP424 (sequence type-ST 924), 063228 (ST 68) and SL066 (ST 429) are located within a type V SCC*mec*, from 49 to 51 kb long, surrounded by insertion sequences (IS), carrying the gene for methicillin-resistance gene *mecA* and one or two copies of the recombinase gene *ccrC* (Fig. 2A). Those three SCC*mec* share at least 98% of identity, which suggests that they have a common origin. Given the fragmentation of some of the other genomes analyzed (strain 2130327043011, for example, is assembled in 114 contigs, while strain MRSP1019 is assembled in 46 contigs), the genetic context of the CRISPR harbored by these strains could not be properly characterized. However, the arrangement and sequences of the spacers of all the aforementioned strains evidence that they indeed have a common origin, since these strains share most of their spacers, in a similar order of organization. The total of 59 spacers carried together by the strains MRSP424, MRSP1019, 063228, 2130327043011 and SL066 correspond to only 19 different sequences (Fig. 2B), with only three of them being exclusive to a strain (spacers 17–19 in *S. pseudintermedius* 2130327043011). Many of these sequences are also common to type III CRISPRs previously described in other *Staphylococcus* isolates (Rossi et al., 2017): *S. schleiferi* TSCC54 (of canine origin), and two strains isolated from human beings, *S. aureus* 08BA02176 and *S. capitis* CR01 (Fig. 2C).

3.4. Transfer of SCCmec from canine to human Staphylococcus strains

The chronological nature of spacer incorporation allowed us not only to infer that the type IIIA CRISPR systems of strains MRSP424, MRSP1019, 063228, 2130327043011 and SL066 have a common origin, but also permitted us to track their transfer processes, which may have involved other strains and/or species, not yet identified or with no available genomes. The spacer sequences indicate that the CRISPR system was transferred through the SCC*mec*, together with the *mecA* gene, between several canine *S. pseudintermedius* strains, here beginning with the 063228 strain, which carries the simplest CRISPR, with a widespread combination of spacers (Fig. 2D). Then, a related

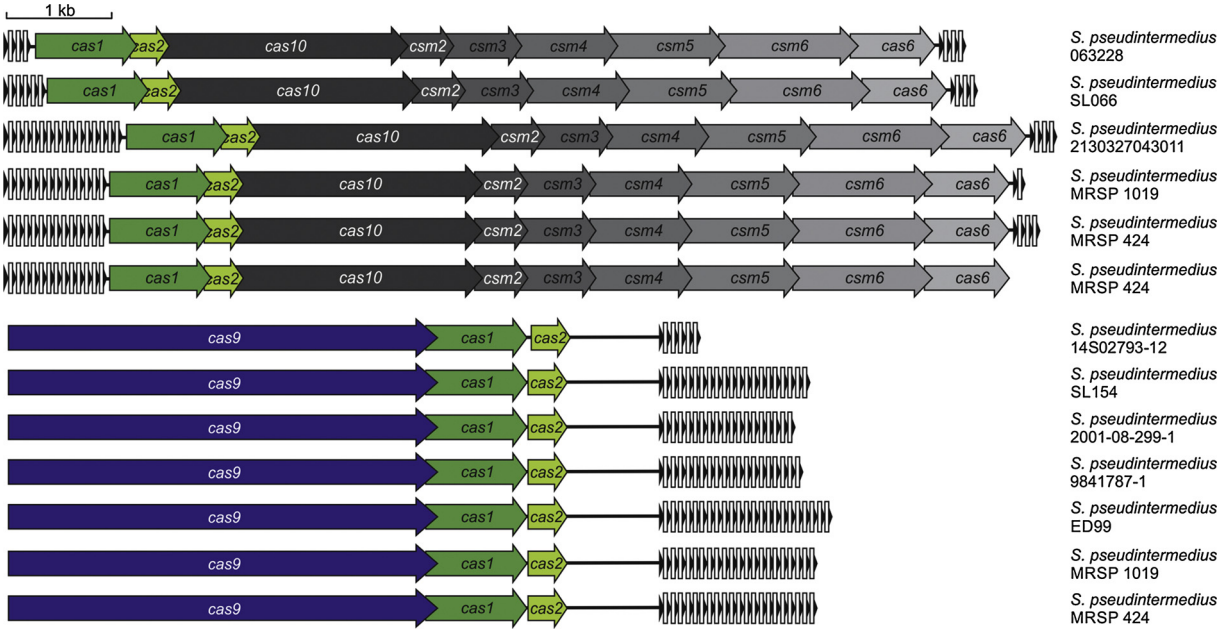

Fig. 1. CRISPR-Cas systems in *Staphylococcus pseudintermedius* strains of canine origin. Two types of systems were found: type II system (with the *cas9* signature gene) and type III (with the *cas10* signature gene). Spacer sequences are represented as white rectangles and direct repeats as black arrowheads.

strain was invaded by elements that led to the incorporation of spacers 7–10, then 9–16. Eventually, the CRISPR that was being shared or transferred only among *S. pseudintermedius* strains, was transferred to an also canine *S. schleiferi* strain. This could be responsible for the spreading of the CRISPR-harboring SCC*mec* to species isolated from human beings, *i.e.*, *S. aureus* 08BA02176 and *S. capitis* CR01. The overall hypothesis of transference is summarized in Fig. 2D.

The CRISPR locus comprised by spacers 4–6, downstream the *cas* genes, seems to be less active than the upstream, since not only no new spacers were acquired in that position, but some were also lost (either completely in *S. pseudintermedius* 2130327043011 and *S. capitis* CR01 or partially in *S. pseudintermedius* MRSP1019). Although not all the pieces of this puzzle are known (since more strains may be involved in the process of horizontal gene transfer), the spacers organization evidence that the SCC*mec* was transferred between different strains, species and hosts. Strikingly, these strains were isolated all over the world: strains 063228, MRSP424 and MRSPA1019 are from the USA, SL066 from Sri Lanka, 2130327043011 from Netherlands, TSCC54 from Japan, 08BA02176 from Canada and CR01 from France.

3.5. CRISPR analysis to assess strain proximity

None of the type IIC CRISPR systems analyzed in here is surrounded by insertion sequences or is located within mobile genetic elements, so their origin is unclear and their horizontal transfer is unlikely. Therefore, strains with similar spacers would probably have a common origin. With exception of strains 14S02793-12 and 2001-08-299-1, whose type IIC CRISPR sequences have no similarities with any other available, the remaining CRISPRs share spacers between at least two strains. The *S. pseudintermedius* strains 98417871 (ST 990) and ED99 (ST 25), for example, share 12 of the 30 (40%) of their spacers (spacers numbered in Fig. 3A as 27–29, 32–39 and 41) organized in a very similar sequence. The same is observed for MRSP424 (ST 924), MRSP1019 (ST 930) and SL154 (ST 121): although the three together share only 3 of the 39 (8%) of their total amount of spacers, strains MRSP1019 and SL154 share 19 of the 22 (86%) of their spacers (Fig. 3B). The origin of these CRISPRs does not seem to be recent, since the strains are all of different MLST types. Again, the area of isolation of each strain show that microorganisms of a common origin were dispersed globally, since the 98417871 strain was isolated from Denmark, ED99 from the UK, MRSP424 and MRSP1019 from the USA, and SL154 from Sri Lanka.

4. Discussion

It is suggested that domestic pets can participate in the cross-transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) within households (Morris et al., 2012), which is consistent with the fact that dogs and their owners may share members of their microbiota when in close contact (Song et al., 2013). This may also explain why some species typically found in dogs' microbiota may eventually be isolated from human beings (Becker et al., 2014). This includes the *Staphylococcus pseudintermedius* species (Devriese et al., 2005), that can be easily misidentified as *S. aureus* or *S. intermedius* in routine diagnostic veterinary tests, and has been reported to be involved with infection in a human being (Van Hoovels et al., 2006). Although these infections are rare, studies suggest that within the *Staphylococcus* genus, species commonly recognized as harmless residents of human beings' or animals' microbiota may act as reservoirs of antimicrobial resistance genes to more virulent species, such as *S. aureus*, thereby increasing their potential to resist the available therapies (Cafini et al., 2016; Rossi et al., 2018).

To what extent this exchange of genetic material may occur *in vivo* is still unclear, but we have recently observed the presence of an almost identical SCC*mec* in a canine *S. schleiferi* and in a *S. aureus* and a *S. capitis* strain isolated from human beings (Rossi et al., 2017). That study was limited to coagulase-negative species that had already been isolated from human beings. In this work, we extended our analyses to canine *Staphylococcus* strains, to better understand the role of domestic dogs in human health as a source of antimicrobial resistance genes.

The genomes available analyzed comprise the species most often isolated from domestic dogs, especially *S. aureus* and *S. pseudintermedius* (Rossi et al., 2018; Wedley et al., 2014). As observed for other staphylococci of human origin (Li et al., 2016; Rossi et al., 2017), CRISPR systems are rarer in staphylococci of canine origin, as the 8% positive strains found in here is much lower than the 50% expected from bacteria in general (Sorek et al., 2008). This is, however, consistent with the abovementioned role that these bacteria may have as reservoirs of

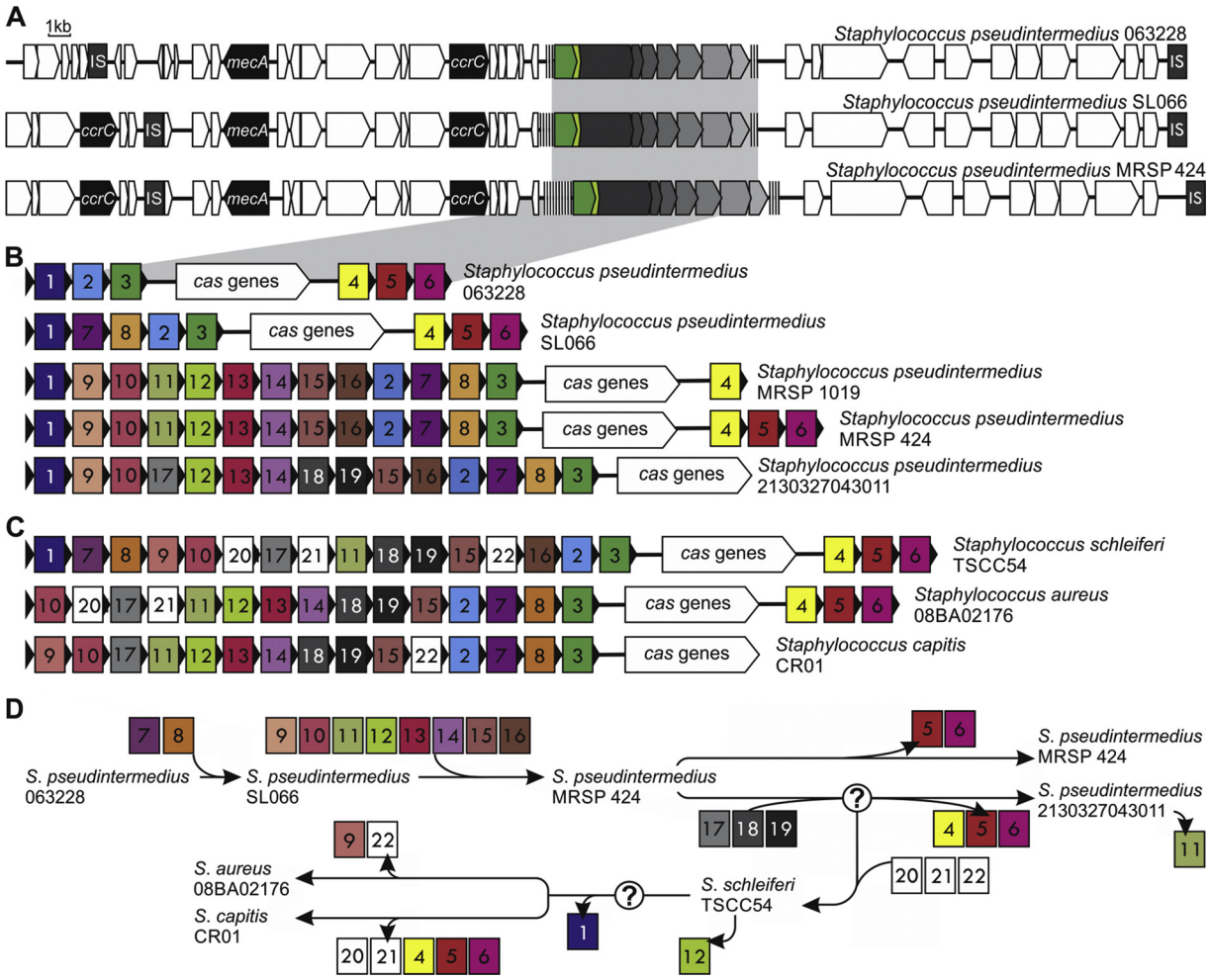

Fig. 2. Type III CRISPR systems features in *Staphylococcus pseudintermedius* of canine origin.(A) Some CRISPR systems are located within a *Staphylococcal cassette chromosome mec* (SCC*mec*), surrounded by insertion sequences (IS) and carryng the methicillin resistance gene *mecA*. (B) Some spacers' sequences and organization are conserved among the strains of *S. pseudintermedius* and (C) are also found in previously characterized CRISPR systems of other species. (D) The spacer sequences and organization suggest the dynamics of the horizontal tranfer of the SCC*mec* between strains and different staphylococcal species. Question marks designate possible intermediate strains. Spacers are represented by numbered squares; those represented by the same color and numered have an identical sequence.

antimicrobial resistance genes and explains why they may exchange genetic material so frequently.

Although at least six types and several subtypes of CRISPR systems are recognized (Luo et al., 2016), only types II and III have been described in staphylococci to date. The fact that only 22% of their spacers map to known sequences evidence that mobile genetic elements, such as plasmids and bacteriophages are still undersampled in the public databases. Still, the existence of spacers of plasmidial origin, especially plasmids that had been described in other *Staphylococcus* species, indicate the failed attempt of plasmid exchange between different strains within the same genus. This evidences that the presence of CRISPR systems indeed interfere with horizontal gene transfer among staphylococci. Even though no significantly significant difference in the number of resistance genes was observed for CRIPR-lacking or CRISPR–carrying strains, this does not invalidate the antiplasmid activity of CRISPR systems, as it has been experimentally demonstrated for *Staphylococcus* strains before (Hatoum-Aslan et al., 2014; Marraffini and Sontheimer, 2008). It is possible that the systems found in the canine strains were acquired (as they can be mobile) after resistance genes' assimilation. Moreover, there is not enough epidemiological data

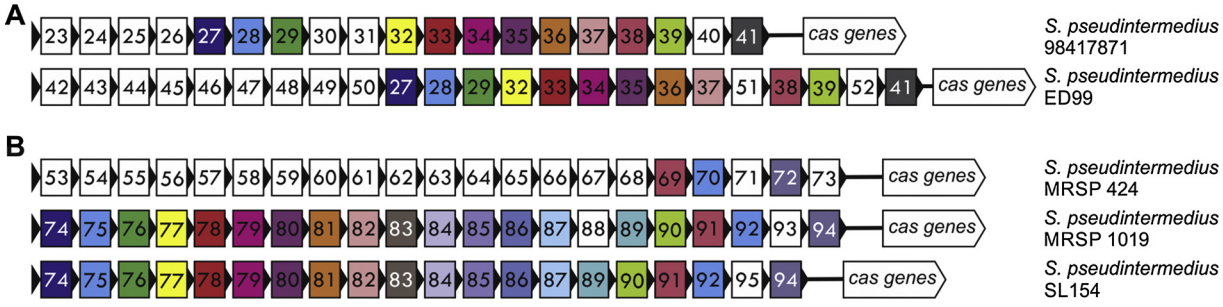

Fig. 3. Type II CRISPR systems features in *Staphylococcus pseudintermedius* of canine origin. Spacer sequences, which are identical when represented by squares of the same color and with the same number, indicate the proximity of (A) strains 98417871 and ED99 and (B) strains MRSP424, MRSP1019 and SL154.

on the origin and the antibiotic therapy to which these strains have been subjected, so they could be properly compared.

Despite of the low frequency of CRISPRs in the strains studied in this work, their presence in some strains allowed us to use their sequence as a molecular clock to track the dynamic process of horizontal gene transfer involving a SCC*mec*. The ordered nature of spacer acquisition suggests that a *S. pseudintermedius*, from the genomes available, was one of the first to acquire and then spread this genetic mobile element, carrying together with the CRISPR the gene for methicillin resistance, *mecA*. Minor alterations in the order of spacers may be the result of homologous recombination between the identical sequences of the direct repeats. In addition, when a *cas* operon is surrounded by two CRISPR loci, one of them is apparently more active than the other (Staals et al., 2016), which explains the loss of spacers in some strains in the less active locus.

Both the CRISPRs carried by a SCC*mec* and the CRISPRs outside a mobile genetic element show that these sequences are a refined and powerful tool to analyze differences between related strains or to track the history of horizontal gene transfer among staphylococci. More importantly, this work shows genetic evidence for the global spreading of pathogenic bacteria and the antimicrobial resistance genes carried by them. Our findings reinforce that the intimate contact between humans and their pets is a potential source of exchange and transportation of microbiota and genetic material. For that reason, in the age of antimicrobial resistance, it is urgent that drug therapies consider the integrated nature of this relationship.

Conflicts of interests

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.04.009.

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