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Carriage of *Staphylococcus aureus* by Free-Living Wild Animals in Spain

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The presence of methicillin-susceptible *Staphylococcus aureus* (MSSA) was analyzed in different free-living wild animals to assess the genetic diversity and predominant genotypes on each animal species. Samples were taken from the skin and/or nares, and isolates were characterized by *spa* typing, multilocus sequence typing (MLST) and antimicrobial susceptibility testing. The proportion of MSSA carriers were 5.00, 22.93, 19.78, and 17.67% in Eurasian griffon vulture, Iberian ibex, red deer, and wild boar, respectively ($P = 0.057$). A higher proportion of isolates ($P = 0.000$) were recovered from nasal samples (78.51%) than skin samples (21.49%), but the 9.26% of red deer and 18.25% of wild boar would have been undetected if only nasal samples had been tested. Sixty-three different *spa* types were identified, including 25 new *spa* types. The most common were t528 (43.59%) in Iberian ibex, t548 and t11212 (15.79% and 14.04%) in red deer, and t3750 (36.11%) in wild boar. By MLST, 27 STs were detected, of which 12 had not been described previously. The most frequent were ST581 for Iberian ibex (48.72%), ST425 for red deer (29.82%), and ST2328 for wild boar (42.36%). Isolates from Eurasian griffon vulture belong to ST133. Host specificity has been observed for the most frequent *spa* types and STs ($P = 0.000$). The highest resistance percentage was found against benzylpenicillin (average, 22.2%), although most of the *S. aureus* isolates were susceptible to all antimicrobial tested. Basically, MSSA isolates were different from those MRSA isolates previously detected in the same animal species.

Staphylococcus aureus is a commensal microorganism in animals and humans (1) that colonizes mainly the nares but also the throat and skin (2–4). *S. aureus* is also the causative agent of several diseases, such as pneumonia and wound and bloodstream infections (3), and colonization has been associated with clinical infection (5, 6).

Methicillin-resistant *S. aureus* (MRSA) emerged by the integration of resistance mechanisms in methicillin-susceptible *S. aureus* (MSSA) (7, 8). The acquisition of *mecA* (9) or *mecC* (10) is a public health concern due to limited options for treatment. Moreover, MRSA infections are related to longer hospitalization stays and higher mortality (11, 12).

MRSA have been detected in domestic animals (13, 14). The genetic background and antimicrobial resistance of *S. aureus* have been associated with host specificity in livestock (8, 15, 16). Companion animals are normally colonized by human-related genotypes, although some studies have described colonization factors that determine host specificity (8, 13). MRSA detection in free-living wild animals in Spain has revealed a very low prevalence but genotypes related to livestock and humans (17, 18). The genetic diversity of MSSA has been studied in domestic animals, also revealing predominant genotypes in different hosts (19, 20). However, little is known about the molecular epidemiology of the susceptible *S. aureus* population in free-living wild animals. In the present study, we investigated the presence of MSSA in free-living wild animals (Eurasian griffon vulture, Iberian ibex, red deer, and wild boar) to assess the genetic diversity and predominant genotypes of MSSA on each animal species. This study would generate new knowledge concerning niches of *S. aureus* that have not been previously investigated.

MATERIALS AND METHODS

Sampling. Apparently healthy animals were captured (box trapping) or hunted between March 2009 and November 2011 in 10 different Spanish provinces. Animals were sampled with sterile swabs through the nares and/or swabbing on ~1 cm^2 of skin (ears or inguinal-mammal area). In total, 2,230 samples from 1,183 animals were tested, including Eurasian griffon vulture (*Gyps fulvus*), Iberian ibex (*Capra pyrenaica*), red deer (*Cervus elaphus*), and wild boar (*Sus scrofa*). The number of individuals per animal species, and the numbers of samples tested are shown in Table 1. Sampling details were previously described (17).

Isolation and identification. Recovery of *S. aureus* (MSSA) was achieved by direct plating on Baird Parker agar with rabbit plasma fibrinogen (bioMérieux) to obtain black colonies with an opaque halo around them as presumptive *S. aureus*. One colony per sample was confirmed as MSSA (*S. aureus mecA* and *mecC* negative) by PCR as previously described (21).

Molecular characterization. All confirmed *S. aureus* isolates were characterized by *spa* typing sequencing the variable fragment of protein A (22). In order to examine further the variation in *spa* types identified, the *spa* types were analyzed by the minimal spanning tree algorithm (Bionumerics 6.0) (Fig. 1). Multilocus sequence typing (MLST) was performed accord-

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TABLE 1 *S. aureus* detection and characterization by *spa* typing and MLST

Animal species	No. of animals		No. of samples/no. of isolates		MLST[a]	*spa* type(s)[b] (no. of isolates)
	Tested	Positive	Nasal samples	Skin samples		
Eurasian griffon vulture	40	2	40/2	0/0	ST133	t7304 (2)
Iberian ibex	157	36	157/36	103/3	ST5	t002 (4)
					ST130	t1736 (3)
					ST425	t3369 (1)
					ST581	t528 (16), t843 (2), t1535 (1)
					ST2328	t3750 (1), **t11501** (1)
					ST2637	**t11221** (7)
					ST2639	t7229 (1), **t11216** (1)
					ST2673	t528 (1)
Red deer	273	54	269/49	273/8	ST1	t098 (1), t127 (2), **t11223** (1)
					ST5	t548 (9), **t11210** (1)
					ST30	t342 (1)
					ST133	t2678 (2)
					ST350	**t11215** (5)
					ST398	t571 (1)
					ST425	t1077 (1), t6386 (1), t6909 (1), **t11208** (3), t11212 (8), **t11228** (1), **t11231** (2)
					ST522	t528 (1), t1534 (1), t3576 (3)
					ST2640	t742 (2)
					ST2671	**t11211** (2), **t11226** (3), **t11233** (3)
					ST2681	t015 (1), **t11217** (1)
Wild boar	713	126	694/103	694/41	ST1	t098 (2), t127 (5), t607 (2), t1407 (2), t2601 (1), **t11223** (1)
					ST5	t548 (18), t2516 (2), t7174 (2), **t11210** (5), **t11214** (1), **t11219** (3)
					ST15	t084 (1)
					ST96	**t11218** (1)
					ST130	t6220 (1)
					ST133	t3583 (7), t10476 (2), **t11220** (1)
					ST188	t189 (2)
					ST398	t034 (2)
					ST425	t742 (3), t6909 (1), **t11222** (1), **t11225** (1), **t11232** (1)
					ST1643	t10712 (7)
					ST2328	t3750 (52), **t11227** (1), **t11230** (8)
					ST2641	**t11229** (1)
					ST2672	t359 (1)
					ST2675	**t11209** (2)
					ST2678	**t11502** (1)
					ST2681	t015 (1)
					ST2682	t6384 (1)
					ST2729	t011 (1)

[a] ST, sequence type. At least one isolate per *spa* type per host reservoir was selected to perform MLST ($n = 88$), the rest being inferred.
[b] Partially published previously (37). New *spa* types and STs identified in the present study are indicated in boldface.

ing to the protocol of Enright et al. (23), with the exception of self-designed primers for *arc*, *tpi*, and *yqiL* housekeeping genes (18). MLST was performed on representative isolates from all *spa* types found per host. At least one isolate per *spa* type and host, together with *S. aureus* with *spa* types containing less than three repeats (24), were characterized by MLST ($n = 88$). Based on these results, sequence types (STs) were assigned.

Phenotypic antimicrobial resistance. Isolates were also tested for antimicrobial susceptibility by broth microdilution (20). Briefly, the MIC was determined by microdilution using Sensititre *Staphylococcus* plate EUST (Trek Diagnostic Systems) and interpreted according to the epidemiological cutoffs established by the European Committee on Antibiotic Susceptibility Testing (http://www.eucast.org/). The antimicrobials tested are shown in Table 2. Only one isolate per animal with the same *spa* type was tested for antimicrobial susceptibility.

Statistical analysis. Comparison of the proportions of positive animals among Eurasian Griffon vultures, Iberian ibex, red deer, and wild boars was performed. A Pearson chi-squared test and the confidence intervals (CIs) at 95% were calculated by using SPSS 20 software and an online tool developed by WinEpi (http://www.winepi.net/sp/disease/cprev1.asp), respectively. Detection of *S. aureus* in nasal samples and skin samples was compared by using the McNemar test (SPSS 20).

Simpson's index of diversity (SID) and Jackknife 95% CI pseudovalues were used to estimate the genetic diversity of MSSA isolates based on *spa* types and MLST (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool) (25), except for Eurasian griffon vultures due to the limited number of *S. aureus* isolates.

A Fisher exact test (SPSS 20) was calculated to analyze the relationship between hosts and *spa* types and STs. Comparison was performed for the

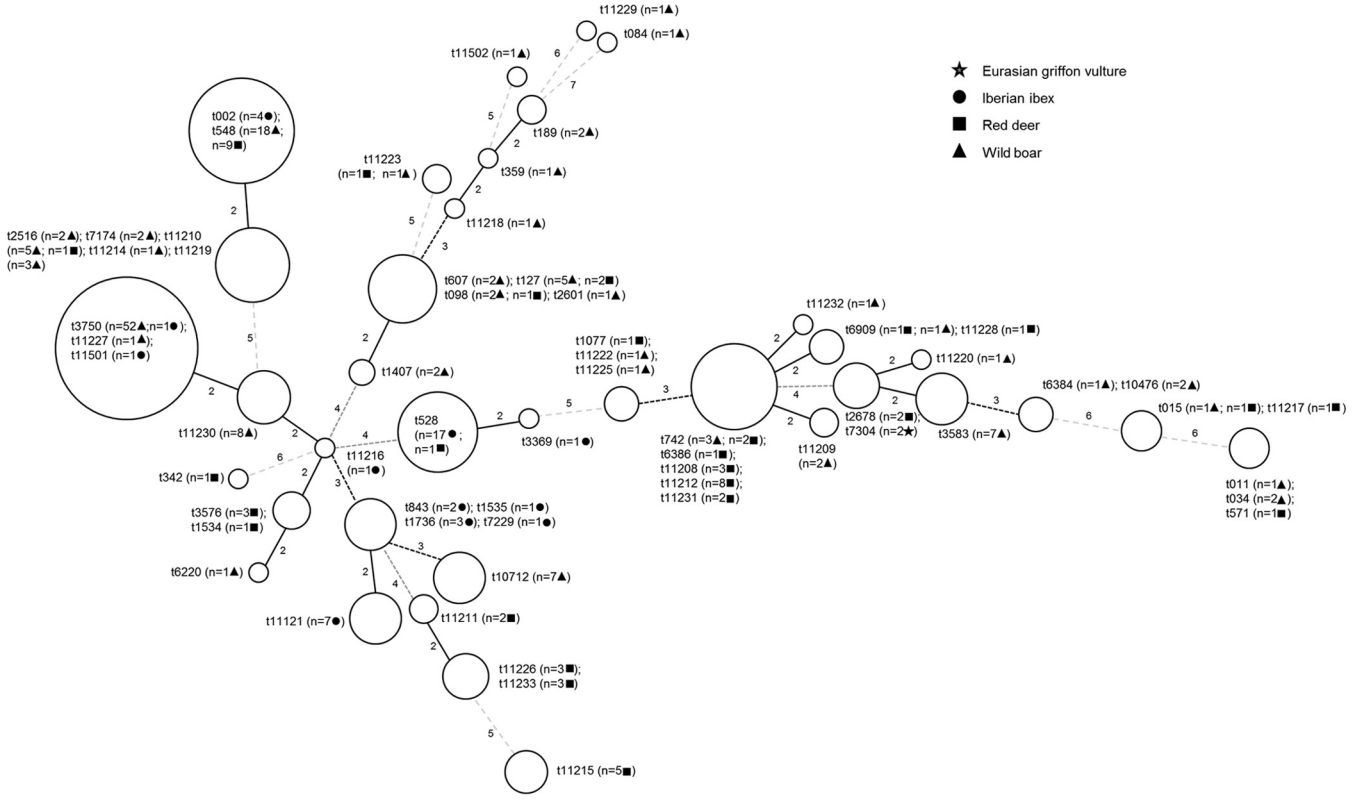

FIG 1 Clustering of *spa* types by minimal spanning tree algorithm. Circles grouped related *spa* types, and the sizes are proportionate to the number of isolates identified for each group. Lines between circles represent the distance coding between the different *spa* types (maximum neighbor distance, 1.00).

most frequent ones in the collection (>5% of the isolates) except for Eurasian griffon vultures because of the limited number of *S. aureus* isolates. The proportion of phenotypic resistance to antimicrobials was compared in Iberian ibex, red deer, and wild boars using the Fisher exact test (SPSS 20).

RESULTS

In total, 242 MSSA isolates were obtained (Table 1). Animals were considered positive regardless of whether *S. aureus* was isolated from skin or nasal samples. The proportion of positive animals was 5.00% (95% CI = 0.00 to 11.75) in Eurasian griffon vultures (2 positive animals out of 40 tested), 22.93% (95% CI = 16.35 to 29.51) in Iberian ibex (36/157), 19.78% (95% CI = 15.05 to 24.51) in red deer (54/273), and 17.67% (95% CI = 14.87 to 20.47) in wild boars (126/473). No significant differences between species ($P = 0.057$) were detected.

A higher proportion of isolates ($P = 0.000$) were recovered from nasal samples (78.51%; 190/242) than from skin samples (21.49%; 52/242) in all animal species where comparison was possible (all except vultures). In red deer and especially in wild boars, double sampling increased the proportion of positive animals detected. If only nasal samples had been tested, five positive red deer (5/54; 9.26%) and 23 positive wild boars (23/126; 18.25%) would have been overlooked. In Iberian ibex, the three positive animals in skin samples were also positive in nasal samples. Half of the animals ($n = 12$) with both nasal and skin samples that were positive (3 Iberian ibex, 3 red deer, and 18 wild boars) presented the same *spa* type in both samples. The other 12 (3 red deer and 9 wild boars) presented different *spa* types.

Sixty-three different *spa* types were identified, including 25 *spa* types not yet reported (Table 1). Distribution of *spa* types is shown in the Fig. 1, where 41 *spa* types clustered in 14 complexes ($n = 169$ isolates) and 22 *spa* types ($n = 73$) not grouped. The most common *spa* types detected were t528 (17/39; 43.59%) in Iberian ibex, t548 (9/57; 15.79%) and t11212 (8/57; 14.04%) in red deer, and t3750 (53/144; 36.11%) in wild boars. The two isolates from Eurasian griffon vulture belonged to *spa* type t7304 (Table 1). In general, *spa* types were mostly detected only in a single host (Table 1). One exception would be t548, which was the most frequent *spa* type detected in red deer and the second most frequent *spa* type in wild boars (Table 1). However, the differences between the most frequent *spa* types and hosts were statistically significant ($P = 0.000$).

MLST analysis yielded 27 different STs, 12 of which had not been described previously (Table 1). The most common STs were ST581 for Iberian ibex (19/39; 48.72%), ST425 for red deer (17/57; 29.82%), and ST2328 for wild boar (61/144; 42.36%). Isolates from Eurasian griffon vultures belonged to ST133 (Table 1). As described above for *spa* types, STs were mostly found in a single host, although some of them (ST1, ST5, and ST133) were sporadically detected in more than one host (Table 1). One exception would be ST425 (Table 1), which was detected mostly in red deer ($n = 17$) but also in wild boar ($n = 7$) and Iberian ibex ($n = 1$).

TABLE 2 Antimicrobial susceptibility and resistance of *S. aureus* isolates using the indicated cutoff value

Antimicrobial	Value (μg/ml)[a]		No. of resistant isolates/no. of tested isolates[b]			
	Cutoff	Tested range	Eurasian griffon vulture ($n = 2$)	Iberian ibex ($n = 36$)	Red deer ($n = 57$)	Wild boar ($n = 135$)
Benzylpenicillin	0.125	0.12–2	0/2	**4/36**	**11/57**	**36/135**
Cefoxitin	4	0.5–16	0/2	0/36	0/57	0/135
Chloramphenicol	16	4–64	0/2	0/36	0/57	**1/135**
Ciprofloxacin	1	0.25–8	0/2	0/36	0/57	0/135
Clindamycin	0.25	0.12–4	0/2	0/36	0/57	0/135
Erythromycin	1	0.25–8	0/2	0/36	0/57	0/135
Fusidic acid	0.5	0.5–4	0/2	0/36	0/57	0/135
Gentamicin	2	1–16	0/2	0/36	0/57	0/135
Kanamycin	8	4–64	0/2	0/36	0/57	0/135
Linezolid	4	1–8	0/2	0/36	0/57	0/135
Mupirocin	0.5	0.5–2,256	0/2	0/36	0/57	0/134
Quinupristin-dalfopristin	1	0.5–4	0/2	0/36	0/57	0/135
Rifampin	0.032	0.016–0.5	0/2	0/36	0/57	0/135
Streptomycin	16	4–32	0/2	0/36	**5/56**	**15/133**
Sulfamethoxazole	128	64–512	0/2	**1/33**	**1/53**	0/135
Tetracycline	1	0.5–16	0/2	0/36	0/57	**4/135**
Tiamulin	2	0.5–4	0/2	0/36	0/57	0/135
Trimethoprim	2	2–32	0/2	0/36	**2/57**	**1/135**
Vancomycin	2	1–16	0/2	0/36	0/57	0/135

[a] The range of studied concentrations is indicated. Cutoff values were as reported online (http://www.eucast.org/mic_distributions/ [last access, 19 May 2014]).
[b] Boldfacing indicates that phenotypic resistance was detected. (The corresponding antimicrobial is also boldfaced in column 1.)

Nevertheless, the differences between the most common STs and hosts were statistically significant ($P = 0.000$).

Based on the *spa* types, the SID was 0.928 (95% CI = 0.907 to 0.949). SID revealed that the genetic diversity was significantly higher ($P < 0.05$) for isolates from red deer (0.913; 95% CI = 0.943 to 0.972) than for isolates from Iberian ibex (0.656; 95% CI = 0.775 to 0.894) and from wild boars (0.794; 95% CI = 0.846 to 0.899) (Table 1). Accordingly, the SID based on MLST was higher for red deer (0.851; 95% CI = 0.797 to 0.905) than for Iberian ibex (0.726; 95% CI = 0.593 to 0.859) and wild boars (0.761; 95% CI = 0.705 to 0.817). Differences were also statistically significant between red deer and wild boars ($P = 0.0207$) but not significant for the comparisons performed for red deer and Iberian ibex or for wild boars and Iberian ibex ($P > 0.05$).

Antimicrobial susceptibly testing revealed that the most of the isolates were susceptible to all antimicrobial tested (Table 2). Nonsignificant differences were detected between the proportion of resistance in isolates from Iberian ibex, red deer, and wild boar ($P > 0.050$) for any of the antimicrobials tested. The most noteworthy resistance percentage was found against benzylpenicillin, with 11.11% (95% CI = 0.84 to 21.38) of isolates from Iberian ibex, 19.30% (95% CI = 9.05 to 29.54) of isolates from red deer, and 26.67% (95% CI = 19.21 to 34.13) of isolates from wild boars being resistant to this antimicrobial (Table 2). Isolates with any resistance to antimicrobials in our panel ($n = 62$) presented 11 phenotypic resistance patterns. The most common ones were resistant only to benzylpenicillin (35 isolates out of 230 isolates tested; 15.22%), resistant to benzylpenicillin-streptomycin (10/230; 4.35%), and resistant only to streptomycin (7/230; 3.04%). The remaining resistance patterns were represented only by one or two isolates with a maximum of resistance to three antimicrobials (benzylpenicillin-streptomycin-tetracycline). Most of the isolates with benzylpenicillin resistance ($n = 51$) belonged to ST5 (30/51; 58.82%). A comparison between ST5 and the proportion of benzylpenicillin resistance showed statistically significant differences ($P = 0.000$).

DISCUSSION

In this study, the genetic background of MSSA isolates from wild animals was determined in order to identify predominant genetic lineages on different free-living wild animal species. The carriage of *S. aureus* has been evaluated revealing that *S. aureus* colonization is common in free-living artiodactyls (Table 1). However, the carriage rates detected in the present study are lower than those reported in different domestic animals such as pigs (36%), small ruminants (from 29 to 64%), donkeys (50%), and rabbits (56%) (26–31). In contrast to the results obtained for MRSA (17), the detection rate of MSSA in the Eurasian griffon vulture was lower than in the Iberian ibex, red deer, and wild boar, although the differences were not significant ($P = 0.057$). The lower number of griffon vultures tested might explain the lack of statistical significance.

Most of the animals simultaneously sampled in nares and skin for isolating *S. aureus* were positive only in one sample (174/198; 87.88%), with nasal swabs being the better option for sampling (172/198; 86.87%). Despite the higher rate of detection in nasal samples, some red deer (5/54; 9.26%) and even more wild boars (23/126; 18.25%) would have tested negative if only nasal samples had been tested. Therefore, double sampling (both nares and skin) is recommended in studies dealing with the detection of MSSA carriers. Similar results have been observed for MRSA (17, 32). The *spa* types detected in nasal and skin samples were different in 50% of the positive animals in both samples, indicating that double sampling increases the diversity of *spa* types found. This should be considered an additional benefit when studying the genetic diversity of MSSA.

The most common *spa* types and STs identified in MSSA isolates in this study (Table 1) were host associated ($P = 0.000$),

suggesting host specificity as previously observed by other authors (33, 34). However, some of these *spa* types and STs (and related *spa* types or STs) have been previously isolated in other animal species, although usually at low frequencies. Thus, ST2328-t9857 (a *spa* type related to t3750) was found in sheep in Denmark (28); t3750 was previously described in Spain in 2006, although the host was not recorded (http://spa.ridom.de/ [accessed 19 May 2014]); ST5-t548 was found in humans and in pigs in the United States and in the United Kingdom (35, 36); ST425-t6386 and t742 were found in humans in the United Kingdom (10); ST581 was found in bulk milk of caprine origin in Portugal in 2003 (www.mlst.net [accessed 19 May 2014]); and ST1740 (a single-locus variant of ST581)-t528 was described in small ruminants in Spain (20). Based on the data available on the MLST webpage (www.mlst.net [accessed 19 May 2014]) ST2328, ST425, ST5, and ST133 are distributed worldwide. However, ST581 and related STs (ST1740, ST1758, ST2490, and ST2673) has been detected in countries in the south of Europe, such as Spain, Portugal, and France.

The dispersion of *spa* types shown in Fig. 1 reflects the high genetic diversity found among MSSA isolates. We compared the genetic diversity of MSSA isolates examined in the present study to the MRSA isolates recovered from the same free-living animal species, where MRSA belonged to ST398 (t011 and t1451) and ST1 (t127) (17). The genetic diversity calculated with the SID based on *spa* types was much higher for MSSA (0.928; 95% CI = 0.907 to 0.949) than for MRSA isolates (0.410; 95% CI = 0.041 to 0.780; $P = 0.0052$). This higher genetic heterogeneity observed in MSSA isolates in free-living wild animals agrees with the higher genetic diversity exhibited by MSSA in previous studies performed in Europe with human isolates (7, 37). When comparing SID (based on *spa* types and STs) between the animal species included in the study (all except vultures), the genetic diversity of MSSA detected in red deer was higher than in wild boars and Iberian ibex. However, the justification for such differences remains unclear. The *spa* types and STs detected among MRSA isolates in free-living wild animals (17) were identified only sporadically in MSSA isolates (Table 1). Thus, only seven isolates belonging to genotype ST1-t127 (Table 1) represented the 2.89% of the MSSA isolates. Similarly, only three MSSA isolates (1.24%) belonged to ST398 (Table 1), and none of them belonged to the *spa* types t011 or 1451 detected in MRSA isolates (17). The single t011 isolate (0.41%) was ST2729, a single-locus variant of ST398.

Most of the MSSA isolates from free-living wild animals presented very low proportions of phenotypic resistance, which is probably linked to the absence of selective pressure due to no antimicrobial use in these animals (38). The highest resistance percentage found in our study was against benzylpenicillin (22.17%; 95% CI = 16.81 to 27.54%). Although the origin of this resistance is unknown, previous studies showed that it is broadly disseminated in food animals (20, 26, 27) and humans (39–41). None of the ST1 and ST398 MSSA isolates exhibited the antimicrobial susceptibility patterns observed in MRSA (tetracycline resistance in ST398 and ciprofloxacin susceptibility plus clindamycin-erythromycin-tetracycline resistance in ST1 [17]).

Overall, the results presented here indicate that the MSSA population in the Eurasian griffon vulture, Iberian ibex, red deer, and wild boar differed in genetic diversity (i.e., SID), genotypes, and antimicrobial resistance patterns from MRSA in the same animal species.

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