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Original Article

High Occurrence of Methicillin-Resistant *Staphylococcus aureus* in Horses at Slaughterhouses Compared with Those for Recreational Activities: A Professional and Food Safety Concern?

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Abstract

The epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) in horses and its zoonotic potential is poorly understood. The objective of this study is to provide data on the prevalence and genetic characteristics of MRSA isolated from horses on farms, at racecourses, and at slaughterhouses in Italy, using standard and molecular methods. In addition, we report the prevalence of MRSA in horse handlers. Among 388 horses tested by nasal swabs, 27 (7%) were positive for MRSA ST398 (t011, t899, t1255) and ST1 (t127). The prevalence of MRSA in horses tested at slaughterhouses was significantly higher ($p < 0.001$) compared with those tested on farms and racecourses. Five (7%) out of 67 staff members working in close contact with horses (2 from slaughterhouse, 2 from riding stable, and 1 from racecourse) were carriers of MRSA ST398 (t011, t034) and ST1 (t127). The isolates from horses and humans carried SCC*mec* IVa or V and were *pvl* negative and *pia* positive. All the isolates from both horses and humans were resistant to at least two antimicrobial classes. The circulation of MRSA in horses and in humans working in close contact with them should be considered an emerging public health issue. In fact, it represents a potential risk for people who work in close contact with horses, and for horse meat consumers.

Keywords: MRSA, horses, racecourse, slaughterhouse, humans

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a widespread and well-known pathogen, able to cause serious infections in humans both in hospitals and in the community (Kluytmans-VandemBergh and Kluytmans, 2006). In addition, MRSA clones are associated with farm and companion animals, and their zoonotic potential is well known (Leonard and Markey, 2006). The presence of MRSA in horses has important implications both for veterinary medicine and for public health. In fact, MRSA could be responsible for several types of infections in horses (Weese *et al*., 2006b). The colonization of horses by MRSA could result in infections in humans working closely with them. There are several reports of outbreaks of MRSA infections in horses admitted to veterinary hospitals and in staff working with horses (O'Mahony *et al.*, 2005). The most prevalent genotype isolated from horses was CC398; this genotype is also responsible for infections in humans, suggesting the circulation of MRSA between horses and humans (Van den Eede *et al.*, 2013). The spread of MRSA in horses intended for meat production could result in colonization and infection of slaughterhouse operators, and represents a potential food safety concern (EFSA, 2009). Italy is the EU's biggest horse meat consumer; about 84,063 horses are slaughtered every year and horse meat production is ~21,466 tons per year (ISTAT, 2009). In addition, people in some Italian regions still consume raw horsemeat (www.cibo360.it/alimentazione/cibi/carne/cavallo.htm). The aim of this report was to investigate (1) the prevalence and characteristics of MRSA in horses used for recreational purposes and for meat production from two regions of southern Italy (Apulia and Basilicata), and (2) the prevalence and characteristics of MRSA in humans working in close contact with horses.

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Materials and Methods

Sampling

During 2014–2015, we analyzed 388 nasal swabs from horses and 67 nasal swabs from staff working closely with horses (farmers, stable hands, slaughterhouse workers, etc.).

Horse and human samples collection. Horse and human swabs were collected from the horses intended for meat production and from the workers in two large abattoirs (M1 and M2), and from horses used for recreational activities and from stable workers at 15 farms or riding stables and 19 stables of a large racecourse.

Sampling for horses intended for meat production at the slaughterhouse. The sampling procedure for horses intended for meat was arranged to create homogeneous groups representative of the animals' origin: of the 171 horses tested, 60 were from Spain, 24 from France, 45 from Italy, and 42 from Poland.

The samples were taken from abattoir horses just after stunning.

Detection, identification, and characterization of MRSA

Detection. Each nasal swab was inoculated to Mueller-Hinton broth (BiolifeItaliana, Milan, Italy) supplemented with 6.5% (w/v) NaCl (Sigma-Aldrich, St Louis, MO). After incubation for 24 h at 35°C, each culture was spread onto a MRSA-SELECT® plate (Bio-Rad, Marnes-la-Coquette, France) and incubated at 35°C for 16–42 h (Nahimana *et al.*, 2006). Suspected colonies were identified by conventional methods as *S. aureus*, and characterized by phenotypic and genotypic methods as reported below.

Microbiological confirmation of methicillin resistance and detection of antimicrobial resistance patterns

Oxacillin and cefoxitin disc diffusion test. Oxacillin and cefoxitin disc diffusion susceptibility tests were performed with 1 μg oxacillin and 30 μg cefoxitin discs (Liofilchem s.r.l, Roseto d. A., Italy), following Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2012—oxacillin; CLSI, 2014—cefoxitin).

Agar screening method. The MRSA suspension was inoculated on Oxacillin Salt Screen Agar® (Mueller-Hinton agar containing 4% NaCl and 6 μg oxacillin/mL, BioLife). Plates were incubated at 35°C for 24 h, and any colony that grew on the plate was considered to be methicillin resistant (Shariati *et al.*, 2010).

Antimicrobial susceptibility testing of MRSA. MRSA isolates were tested for susceptibility to a panel of 11 antimicrobial agents using the disc diffusion method on Mueller-Hinton agar (BioLife), following the CLSI guidelines (CLSI, 2013—enrofloxacin; CLSI, 2014—all other antibiotics). The antibiotic discs from Liofilchem were as follows: amikacin (30 μg), clindamycin (2 μg), chloramphenicol (30 μg), doxycycline (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin (10 Units), sulfisoxazole (250 μg), and tetracycline (30 μg).

All these tests were carried out using the ATCC 43300 and ATCC 25923 (Biogenetics) isolates as methicillin-resistant and methicillin-susceptible *S. aureus*, respectively.

Molecular confirmation of methicillin resistance and genetic characterization of MRSA. Genomic DNA was extracted from the presumptive MRSA isolates using the GenomicPrep® Cell and Tissue Isolation Kit (Amersham, Piscataway, NJ) following the manufacturer's instructions.

Real-time polymerase chain reaction. Two separate SYBR Green polymerase chain reaction (PCR) assays were optimized to confirm the species identification and to detect the *mecA* gene, using previously described primers, sau1 and sau2 (Strommenger *et al.*, 2003) and mecA147-F and mecA147-R (Zhang *et al.*, 2005), respectively. One isolate per sample identified as MRSA was further characterized as described below.

Molecular characterization of MRSA. The isolates were further characterized by multilocus sequence typing (MLST), *spa*-typing, and staphylococcal chromosomal cassette *mec* element (SCC*mec*) characterization as specified below.

The PCR amplifications at the seven loci included in the MLST scheme, *arcC*, *aro*E, *glp*F, *gmk*, *pta*, *tpi*, and *yqi*L, were performed as described elsewhere (Enright *et al.*, 2000). The eBURST program was used to determine the group of each sequence type (ST) based on the MLST database. Alleles and the ST were assigned according to *Staphylococcus* MLST database (http://saureus.mlst.net).

The x region of the *spa* gene was amplified by PCR using primers spa-1113f and spa-1514r (Strommenger *et al.*, 2006). *spa*-types were determined using BioNumerics 7.6 software (Applied Maths) according to Ridom spa Server (http://spa.ridom.de/index.shtml).

SCC*mec* typing was carried out as described by Zhang *et al.* (2005).

All the MRSA isolates were tested by PCR for *luk*S-*luk*F-PV, encoding Panton-Valentine leukocidin (PVL) (Hesje *et al.*, 2011), for the *ica*A gene (intercellular adhesion) (Zmantar *et al.*, 2008), and for *sea* to *sej* and *sem* to *seo* encoding staphylococcal enterotoxins (SEs) (Boerema *et al.*, 2006) as described elsewhere.

Statistical analysis. Differences among the percentages of MRSA detected in horse and human samples were compared using the Chi-square test (χ^2, $p < 0.05$) with Epi Info 3.3.2 software.

Results

Prevalence of MRSA in horses and humans

Out of 388 horse nasal swabs examined, 27 (7%) were positive for MRSA; 26 (96%) of which came from horses sampled in slaughterhouses and 1 (4%) from a horse at a riding stable.

The isolation rates of MRSA was statistically higher in slaughtered horses (26/171–15%) compared with those sampled on farms or at riding stables (1/147–1%) ($p < 0.001$) and racecourses (0/70–0%) ($p < 0.001$) (Table 1).

Table 1. Detection of Methicillin-Resistant *Staphylococcus aureus* in Horses

Sampling site	*No. of horses*	*No. of positive (%)*
Slaughterhouse	171	26 (15)
Farm/riding stable	147	1 (1)
Racecourse	70	0 (0)
Total	388	27 (7)

Table 3. Detection of Methicillin-Resistant *Staphylococcus aureus* in Horse Personnel

Sampling site	*No. of humans*	*No. of positive (%)*
Slaughterhouse	16	2 (12)
Riding stable	37	2 (5)
Racecourse	14	1 (7)
Total	67	5 (7)

Among the MRSA-positive horses from slaughterhouses, 76.9% (20/26) were from Spain, 19.2% (5/26) from Poland, and 3.85% (1/26) from Italy (Table 2). None of the positive horses came from France. The isolation rate in Spanish horses (20/60–33.3%) was significantly higher than that registered for Polish (5/42–11.9%) ($p < 0.001$), Italian (1/45–2.2%) ($p < 0.001$), and French (0/24–0%) horses ($p < 0.001$) (Table 2). Furthermore, the isolation rate in Polish horses (5/42–11.9%) was significantly higher than that observed in Italian (1/45–2.2%) ($p < 0.01$) and French (0/24–0%) horses ($p < 0.001$). Five (7.5%) of the 67 analyzed swabs from people working closely with horses tested positive for MRSA. Of the positive swabs, 2/5 were from slaughterhouse staff, 2/5 from breeders, and 1/5 from racecourse operators (Table 3).

The isolation rates of MRSA was higher in slaughterhouse operators (2/16–12%) than in the operators of riding stables (2/37–15%) and of racecourses (1/14–7%); however, only isolation rates in slaughterhouse staff and in operators of riding stables resulted statistically different ($p < 0.05$).

Antimicrobial resistance pattern of MRSA isolates from horses and humans

The results from the antimicrobial susceptibility tests performed on MRSA isolated from horses and humans are reported in Tables 4 and 5.

Genotyping of MRSA isolates from horses and humans

Tables 4 and 5 show the results of genotyping (*spa*-typing, MLST, SCC*mec* typing, PVL, PIA, and SEs encoding genes) performed on MRSA isolated from horse and human nasal swabs, respectively.

Genotyping analysis by MLST and SCC*mec* typing of 27 MRSA isolated from horses identified the following genotypes, ST398/IVa/V (26/27–96%) and ST1/IVa (1/27–4%). ST398 isolates showed the following *spa*-types: t011 (20/26–77%), t899 (5/26–19%), and t1255 (1/26–4%). ST1 isolates showed *spa*-type t127. The 20 ST398-t011 isolates came from horses slaughtered in abattoir M1: 19 from Spanish horses and 1 from an Italian horse. The ST398-t1255 isolate was detected in a riding stable horse and five ST398-t899 isolates in Polish horses slaughtered in the abattoir M2 (Table 4).

Among the five human isolates, the following genotypes were detected: ST398/V (3/5–60%) and ST1/IVa (2/5–40%). ST398 isolates showed the following *spa*-types: t011 (1/3–33%) and t034 (2/3–77%). Both ST1 isolates showed *spa*-type t127 (Table 5).

Both ST398-t034 isolates came from the staff of a farm where MRSA was not isolated in horses. The ST398-t011 isolate came from a slaughterhouse worker (M2); MRSA isolated from horses at the same slaughterhouse were of the ST398-t899 genotype. ST1-t127 isolates came from a racecourse operator and a slaughterhouse worker at M1 (Table 5). While the horse nasal swabs sampled at the racecourse all tested negative for MRSA, 1 of the 20 isolates detected from horses in M1 showed a genetic profile (ST1-t127) identical to the isolates from a worker at this slaughterhouse.

The results of the *mecA*, PVL genes, *icaA* gene, and SE genes are reported in Tables 4 and 5.

Table 2. Methicillin-Resistant *Staphylococcus aureus* in Horses Sampled in Slaughterhouses

Slaughterhouse	*Origin*	*No. of horses*	*No. of positive (%)*
M1	Spain	60	20 (33)
M1	France	24	0 (0)
M1	Italy	45	1 (2.2)
M2	Poland	42	5 (12)
Total	171		26 (15)

Discussion

MRSA is an emerging pathogen in horses. It was previously associated with sporadic infections in horses treated in veterinary hospitals (Weese *et al.*, 2005; Garcia-Alvarez *et al.*, 2012), and its spread in this animal species has now become a global issue (Weese and Van Duijkeren, 2010). The first MRSA isolated from horses belonged to the clonal complex 8 (CC8), a clone of human origin, spread mainly in horses in Canada and the United States (Weese and Van Duijkeren, 2010). Subsequently, an MRSA isolate of CC398, a clone typically associated with livestock, was isolated in horses. The spread of MRSA in horses has been widely investigated in several European countries and in North America, and surveys have been conducted on horses on farms and those treated in veterinary hospitals (Baptiste *et al.*, 2005; Busscher *et al.*, 2006; Weese *et al.*, 2006a; Van den Eede *et al.*, 2009). Although the presence of MRSA in farm animals and in humans in Italy has been widely documented (Pan *et al.*, 2009; Battisti *et al.*, 2010; Normanno *et al.*, 2015), very few investigations have focused on horses and personnel working with them (De Martino *et al.*, 2010; Mallardo *et al.*, 2013; Carfora *et al.*, 2016). In our study, the isolation rate of MRSA recorded in slaughtered horses was significantly higher than in those used for recreational purposes and racehorses, probably due to the rearing system of horses intended for meat production and the higher antimicrobial selective pressure present during rearing stages. In particular,

Table 4. Genotyping and Antimicrobial Resistance Pattern of the Methicillin-Resistant *Staphylococcus aureus* Isolated from Horses

Strain	*Sampling site*	*Origin*	spa *type*	*ST*	*SCC*mec	pvl	icaA	se*(s)*	*AMR-pattern*
1	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DXT-CN-K-OX-P-TE
2	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DA-DXT-CN-K-OX-P-TE
3	Slaughterhouse	Spain	t011	398	IVa	–	+	—	AK-CF-DXT-E-CN-K-OX-P-TE
4	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-CN-K-OX-P-TE
5	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-CN-K-OX-P-TE
6	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-CN-K-OX-P-TE
7	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DXT-CN-K-OX-P-TE
8	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DA-DXT-E-CN-K-OX-P-ST-TE
9	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DA-DXT-E-CN-K-OX-P-ST-TE
10	Slaughterhouse	Spain	t011	398	IVa	–	+	—	AK- CF-DA-C-DXT-E-CN-K-OX-P-ST-TE
11	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-C-DXT-CN-K-OX-P-ST-TE
12	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DA-DXT-E-CN-K-OX-P-TE
13	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DXT-E-CN-K-OX-P-TE
14	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DXT-CN-K-OX-P-TE
15	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DXT-E-CN-K-OX-P-TE
16	Slaughterhouse	Spain	t011	398	IVa	–	+	—	CF-DXT-CN-K-OX-P-TE
17	Slaughterhouse	Spain	t011	398	V	–	+	*g*	CF-DA-C-DXT-ENR-OX-P-TE
18	Slaughterhouse	Spain	t011	398	V	–	+	*g*	CF-DA-C-DXT-ENR-OX-P-ST-TE
19	Slaughterhouse	Spain	t011	398	V	–	+	*g*	CF-DA-C-DXT-ENR-OX-P-ST-TE
20	Slaughterhouse	Spain	t127	1	IVa	–	+	*h*	CF-ENR-E-CN-K-OX-P-TE
21	Slaughterhouse	Italy	t011	398	IVa	–	+	*n, g*	AK-CF-DXT-CN-OX-P-TE
22	Slaughterhouse	Poland	t899	398	V	–	+	—	CF-DXT-OX-P-ST-TE
23	Slaughterhouse	Poland	t899	398	V	–	+	—	AK-CF-DA-DXT-ENR-E-CN-K-OX-P-TE
24	Slaughterhouse	Poland	t899	398	V	–	+	—	CF-C-DXT-CN-OX-P-ST-TE
25	Slaughterhouse	Poland	t899	398	V	–	+	—	CF-DA-C-DXT-E-K-OX-P-ST-TE
26	Slaughterhouse	Poland	t899	398	V	–	+	—	CF-DA-DXT-OX-P-TE
27	Riding stable	Italy	t1255	398	V	–	+	—	CF-DXT-OX-P-TE

AK, amikacin; C, chloramphenicol; CF, cefoxitin; CN, gentamicin; DA, clindamycin; DXT, doxycycline; E, erythromycin; ENR, enrofloxacin; K, kanamycin; OX, oxacillin; P, penicillin; SCC*mec*, Staphylococcal cassette chromosome *mec* element; ST, sulfisoxazole; TE, tetracycline.

the isolation rate recorded in Spanish and Polish horses reflects the high prevalence found in horses slaughtered in abattoirs. In fact, the prevalence of MRSA in French and Italian horses was low, and similar to values recorded in horses used for recreational purposes and in racehorses. To our knowledge, no prevalence data are available on MRSA in horses from Spain and Poland, although the isolation of MRSA in hospitalized horses has been reported once in Spain (Gómez-Sanz *et al.*, 2014). As for the people working closely with horses, the percentage of MRSA isolation was higher in the slaughterhouse staff than in workers of farms, riding stables, and racecourses. This finding might be associated with slaughterhouse staff being more exposed to MRSA and to a higher level of MRSA contamination than workers at farms, riding stables, and racecourses. In fact, it is widely reported that when the prevalence of MRSA in animals is high, people in close contact with them are at a much greater risk of colonization and subsequent infection (EFSA, 2009). The antimicrobial susceptibility tests have shown that human and horse isolates are resistant to a wide range of antimicrobials of different families. Therefore, they have the characteristics of multiresistant isolates, similar to what has been reported for MRSA isolates of animal and human origin worldwide (Weese and van Duikeren, 2010). Regarding genetic characterization of the isolates, ST398 is the prevalent genotype in horses and in staff working closely with them. MRSA isolates belonging to ST398 are the most widespread in Europe in horses; ST398/t011 strains were isolated in

Table 5. Genotyping and Antimicrobial Resistance Pattern of the Methicillin-Resistant *Staphylococcus aureus* Isolated from Staff Working in Close Contact with Horses

Strain	*Sampling site*	spa *type*	*ST*	*SCC*mec	pvl	icaA	se*(s)*	*AMR-pattern*
1	Slaughterhouse	t127	1	IVa	–	+	*h*	CF-E-CN-K-OX-P-ST-TE
2	Slaughterhouse	t011	398	V	–	+	—	AK-CF-DA-DXT-E-CN-K-OX-P-ST-TE
3	Farm	t034	398	V	–	+	—	CF-DA-C-DXT-OX-P-TE
4	Farm	t034	398	V	–	+	—	CF-DXT-OX-P-TE
5	Racecourse	t127	1	IVa	–	+	h, m	CF-E-K-OX-P

AK, amikacin; C, chloramphenicol; CF, cefoxitin; CN, gentamicin; DA, clindamycin; DXT, doxycycline; E, erythromycin; K, kanamycin; OX, oxacillin; P, penicillin; SCC*mec*, Staphylococcal cassette chromosome *mec* element; ST, sulfisoxazole; TE, tetracycline.

Austria, Belgium, Spain, and the Netherlands (Cuny *et al.*, 2008; van Duijkeren *et al.*, 2010; Van den Eede *et al.*, 2012; Gómez-Sanz *et al.*, 2014), and MRSA ST398 is typically associated with livestock (LA-MRSA). These strains have been shown to cause serious infection in humans, mainly in people coming into close contact with animals for work (Cuny *et al.*, 2010; Köck *et al.*, 2013). Handling and/or consumption of food products contaminated by CC398 could be also risk factors for infection (EFSA, 2009). In addition, transmission of a MRSA ST398/t011 from a horse to human has been demonstrated in the Netherlands (van Duijkeren *et al.*, 2011). In slaughterhouses, samples from both horses and workers were MRSA positive, whereas samples taken from the horses at stables and racecourses were MRSA negative, although some of the human nasal swabs tested positive. In our study, human and horse MRSA ST398 were isolated from samples collected at the same sampling point at M2: whereas these isolates presented the same ST and SCC*mec*, they actually belonged to different *spa*-types, therefore transmission from horses to humans or *vice versa* does not seem likely. The other genotype was ST1, identified in three isolates: from a slaughtered Spanish horse, a slaughterhouse operator at M1, and from a racecourse operator; all isolates had *spa*-type t127 and SCC*mec* IVa. MRSA ST1/t127 strains are typically associated with human infections, and cases have been reported in the United States, Germany, and the United Kingdom. In addition, these strains were among the prevalent clones isolated in human cases of invasive MRSA infections in Europe (Otter *et al.*, 2009; Monaco *et al.*, 2013). MRSA ST1/t127 was isolated from horses in different veterinary hospitals (Cuny *et al.*, 2008; Loncaric *et al.*, 2014), and from pigs, cows, sheep, and goats in Italy (Battisti *et al.*, 2010; Cortimiglia *et al.*, 2015; Caruso *et al.*, 2016). In our study, human and horse MRSA ST1 were isolated from swabs taken at the same sampling point at M1. Unlike what was found at slaughterhouse M2, the two isolates showed the same genetic characteristics. Therefore, transmission of the strain from horses to humans and *vice versa* might be plausible.

Regarding the genetic virulence markers detected in our isolates, we can summarize some considerations. All the isolates that carried the *icaA* gene were *pvl* negative and some were potentially enterotoxigenic. This suggests that although the *pvl*-negative isolates are generally less virulent than the Panton-Valentine leukocidin (PVL) producers, these isolates could be of concern for the meat industry. These strains can synthesize a biofilm that promotes resistance and persistence of the strain in the food industry, and produce some SEs, alone or in combination (SEH, SEG, SEM, and SEN). However, the foodborne risk appears quite limited, because the SE most frequently involved in staphylococcal food poisoning (SFP) outbreaks is SEA (Balaban and Rasooly, 2000), and the role of the recently discovered SEs in food safety is largely unknown. On the other hand, SEH-producing strains have been involved in SFP outbreaks (Ikeda *et al.*, 2005), thus, the risk of foodborne intoxication linked to our findings must be carefully assessed. MRSA isolated from humans and animals show great variability in antimicrobial susceptibility, and this is of concern in human and veterinary medicine. In this study, the MRSA isolates from both horses and humans were resistant to between 9 and 13 antimicrobials. In our study, all the ST398 isolates, generally recognized as a livestock-associated clone (LA-MRSA), showed resistance to tetracycline; on the other hand, the only strain susceptible to tetracycline was the ST1/t127/IVa isolated from a human source. This finding reinforces the hypothesis that the excessive use of tetracycline in animal husbandry, especially on pig farms, could lead to the selection of clones carrying resistance (de Neeling *et al.*, 2007).

In conclusion, the results of our study show that while a low MRSA prevalence is recorded in horses and workers in contact with them at farms, riding stables, and racecourses, a high prevalence was found in slaughterhouses, where a high prevalence was detected in imported horses. In addition, the detection in horses of MRSA belonging to the human transmissible genotypes (ST398, ST1) and the isolation of strains with identical genetic profiles in a horse and a worker at the same slaughterhouse, confirm that there is a potential zoonotic risk from direct contact with animals, or from the handling and consumption of raw horse meat, which is a widespread habit in some European countries.

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Disclosure Statement

No competing financial interests exist.

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