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Detection of livestock-associated methicillin-resistant *Staphylococcus aureus* among swine workers in Romania

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Methicillin-resistant *Staphylococcus aureus* (MRSA);
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Summary
Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a devastating pathogen that is associated with high morbidity and mortality worldwide. Livestock are a well-known reservoir for this pathogen, which poses substantial health risks for livestock workers. Little is known about the epidemiology of livestock-associated MRSA (LA-MRSA) among livestock workers in Eastern Europe.
Methods: To study the epidemiology of LA-MRSA among swine workers in Romania, we collected and characterized nasal and oropharygneal samples from swine workers on commercial pig farms. A survey that included questions about work-related tasks, biosafety practices, contact with animals, and health status was used to assess the risk factors that were potentially associated with LA-MRSA colonization.
Results: The prevalence of MRSA colonization among swine workers was 6.8%. Two LA-MRSA strains with the *spa* types t034 and t011 and one likely community-associated MRSA strain with the *spa* type t321 were isolated from workers on five farms. Interestingly, all MRSA carriers worked on farms that imported animals from other production facilities.

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Conclusion: This is the first study to confirm the presence of LA-MRSA among swine workers in Romania and suggests the need to minimize the risk of LA-MRSA-related infections in swine workers and their community contacts. The findings also suggest a link between the commercial movement of swine and the introduction of LA-MRSA.
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Introduction

Staphylococcus aureus is a Gram-positive extracellular bacterium that colonizes up to 36.4% of the population of Europe [1]. Antibiotic-resistant strains, including methicillin-resistant *S. aureus* (MRSA), cause a wide range of skin and soft tissue infections and invasive diseases. Hospital-acquired MRSA (HA-MRSA) infections are estimated to affect more than 150,000 patients in Europe and to cost the European Union (EU) health care system approximately 380 million euros annually [2]. Additionally, infections due to community-acquired MRSA strains (CA-MRSA) have been documented in individuals without any recent exposure to health care [3,4]. Consequently, the EU has identified the prevention and control of MRSA-associated infections in health care facilities and community settings as a top public health priority, and the Centers for Disease Control and Prevention recently listed MRSA as a ''microorganism with a threat level of serious'' in its 2013 Threat Report [5].

MRSA strains associated with livestock (LA-MRSA) have been isolated from pigs, cattle, and poultry in numerous European countries [6—8]. Studies from the Netherlands have demonstrated that living in areas with high livestock densities is a risk factor for MRSA colonization. Additionally, the rate of MRSA infection is substantially higher in hospitals that are located in livestock-dense areas [9,10].

Pigs are the predominant carriers of LA-MRSA and thus swine workers are at an increased risk of MRSA colonization. LA-MRSA strains have been detected in swine production facilities in 17 EU Member States [11], and interspecies transmission of LA-MRSA has also been reported [12,13]. In Europe, 23—38% of individuals who have had contact with MRSA-positive pigs are MRSA carriers, and 4% of their family members, who have not had direct exposure to these animals, are colonized [12]. These studies indicate that regular contact with live pigs is an important risk factor for acquiring LA-MRSA, and we postulate that swine workers play a substantial role in MRSA transmission via the spread of the pathogen to their community contacts.

The LA-MRSA strains carry molecular characteristics that are distinct from those of the HA- and CA-MRSA strains. Many LA-MRSA strains belong to the multilocus sequence type (MLST) clonal complex 398 (ST398) [14] and often lack Panton—Valentine leukocidin (PVL), which is a cytotoxin that is observed in many CA-MRSA strains [15]. Furthermore, LA-MRSA isolates exhibit resistance to many non-beta-lactam antimicrobials that are often used in livestock production, including macrolide, gentamicin, ciprofloxacin, and trimethroprim—sulfamethoxazole [16].

According to the European Antimicrobial Resistance Surveillance Network, Romania has one of the highest rates of invasive MRSA infections in Europe, and this rate has been rising steadily since 2007 (www.rivm.nl/earss/database). The diversity of MRSA strains that have been detected in healthcare facilities is remarkable and indicative of the continuing evolution and transmission of HA-MRSA strains in this country [17]. CA-MRSA strains have also been identified [18].

While Romania is considered a ''hot spot'' for MRSA infections, LA-MRSA has not yet been reported in this country. To investigate the epidemiology of MRSA in commercial swine production in Romania, we conducted a convenience survey of swine workers from several large pig farms in the Transylvania region of Romania. Our survey assessed occupational risk factors and MRSA colonization statuses.

Materials and methods

Sample collection

In June 2012, we invited workers from seven commercial swine farms in the Northwestern region of Romania to participate in our study. Trained Romanian-speaking data collectors used a questionnaire to obtain information about the demographics, work, biosafety practices, contact

with animals, and health statuses of the participants. Nasal and oropharygneal swabs were also collected. All participants provided written informed consent prior to enrolment in the study and received a small stipend after completing the study. The study protocols were approved by the Institutional Review Board of the Yale School of Medicine and the Environmental Health Center at Cluj-Napoca in Romania.

Study sites

Farm A was a farrow-to-finish operation that consisted of multiple buildings with age-segregated nurseries, finishing, and wean-to-finish pigpens. Farms B through G had several buildings that housed only finishing pigs. Environmental samples were collected from randomly chosen wall corners on each farm. Areas of 10 cm × 10 cm, 1 m above the floor, on both sides of the wall, were sampled using sterile swabs. Wall corners were chosen as the sampling sites because they are a source of MRSA where pigs come into contact with often.

Isolation of *S. aureus*

Swabs of the nasal, oropharygneal, and environmental surfaces were stored in 2 mL of transport medium at 4 °C during transportation. The samples were inoculated in 3 mL of Mueller-Hinton broth supplemented with 6.5% NaCl and incubated at 37 °C for 24 h. The samples were then diluted (1:10) in Tryptic Soy Broth containing 3.5 mg/l cefoxitin and 75 mg aztreonam and incubated for 24 h at 37 °C [19]. Single loopful of broth was inoculated onto selective MRSA chromogenic agar plates (Brilliance™ MRSA 2 Agar, Oxoid) and incubated for 48 h at 37 °C. Presumptive MRSA-positive colonies were shipped to a microbiology laboratory at the University of Iowa, United States for further molecular characterization.

Characterization of MRSA isolates

Presumptive positive colonies were streaked onto Columbia CNA with 5% sheep blood (Becton Dickinson and Company, Sparks, Maryland, USA) and incubated for 24 h at 35 °C. All *S. aureus* isolates were confirmed using the catalase test, coagulase test, and Pastorex Staph-plus latex agglutination assay (Bio-Rad, Redmond, Washington, USA). All isolates that tested positive for *S. aureus* were subjected to molecular testing and antimicrobial susceptibility testing.

Antimicrobial susceptibility testing

S. aureus isolates were tested for antimicrobial susceptibility using the minimum inhibitory concentration methodology described by the Clinical Laboratory Standards Institute [20]. The isolates were tested for susceptibility to oxacillin, tetracycline, erythromycin, clindamycin, trimethoprim—sulfamethoxazole, gentamycin, levofloxacin, vancomycin, daptomycin, quinupristin/dalfopristin, linezolid, and rifampin.

Molecular testing

Genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, Wisconsin, USA). Total DNA for plasmid analysis was extracted via heat lysis. The presence of PVL, beta-lactam antibiotic resistant gene *mec*A, florfenicol resistant genes *fex*A and *cfr*, and trimethoprim resistant gene *dfr*K were determined by PCR. All isolates that carried the *mec*A gene were identified as MRSA. *Spa* typing was performed using the primers described by Ridom Bioinformatics (ridom.de/doc/Ridom_spa_sequencing.pdf), and the sequences were interpreted using the Ridom StaphType software (Ridom GmbH, Würzburg, Germany). All molecular procedures employed known positive and negative controls.

Statistical analyses

Univariate analyses were performed to determine the prevalence of MRSA colonization among the swine workers. The potential risk factors associated with MRSA colonization were assessed using the Pearson chi-squared test and Fisher's exact test. Due to the small sample size, descriptive epidemiology was employed to assess MRSA carriage status. The level for significance was set a $p < 0.05$. All statistical analyses were performed using SAS software version 9.3.

Results

Characteristics of the swine farms

The farm characteristics, including the number of pigs, farm type, number of workers, and the origin of the swine population, are shown in Table 1. Farm A was a farrow-to-finish operation that housed approximately 33,000 pigs. Approximately 10,000 finishing pigs were raised on farms B and C, and

Table 1 Characteristics of swine farms, June 2012.

Variables	Farms						
	A	B	C	D	E	F	G
Farm type	Farrow-to-finish	Finish	Finish	Finish	Finish	Finish	Finish
Number of pigs[a]	33,000	11,000	9000	1200	1300	1350	1300
Workers sampled[b]	97.1 (67/69)	63.2 (12/19)	84.6 (11/13)	100.0 (4/4)	100.0 (4/4)	100.0 (3/3)	100.0 (2/2)
MRSA carriers[c]	0.0 (0/67)	0.0 (0/12)	9.1 (1/11)	50.0 (2/4)	25.0 (1/4)	66.7 (2/3)	50.0 (1/2)
Country of pig origin	Romania	Romania	Romania	Germany, Netherlands, Denmark, Slovakia	Hungary, Slovakia	Germany, Netherlands, Denmark, Slovakia	Germany, Netherlands, Denmark, Slovakia

[a] Estimated number of pigs on the farm in June 2012.
[b] Table values are percentage of workers sampled (number sampled/total number of workers on the farm).
[c] Table values are percentage of MRSA carriers (number of carriers/number sampled).

1300 finishing pigs were raised on farms D, E, F, and G. Farm A also had the greatest number of workers ($n = 69$), followed by farm B ($n = 19$) and farm C ($n = 13$). Farms D through G each had fewer than five workers.

Prevalence of MRSA colonization in the workers

A total of 103 workers provided both nasal and oropharyngeal swabs. More than 80% of the workers from farms A and C were sampled for MRSA, and 63% of the workers from farm B were sampled. All of the workers on farms D through G were sampled for this study (Table 1).

Among the 103 workers who provided nasal and oropharyngeal samples, seven participants (6.8%) from five farms tested positive for MRSA. Two were colonized in both the nares and oropharynx, two were colonized in the oropharynx only, and three additional workers were colonized only in the nares. The prevalence of human MRSA carriage on these farms was 0—67%. No identified cases of MRSA were found on the largest farm, which was a farrow-to-finishing farm that did not import pigs from other swine facilities. Higher prevalence rates of MRSA were observed on farms that routinely imported finishing pigs from foreign countries (Table 1).

Characteristics of the MRSA isolates

Twenty-seven human isolates from 25 workers were shipped to the laboratory in the US. Among these, nine human isolates from seven workers carried the *mec*A gene (MRSA-positive). Three *spa* types, including t011, t034, and t321, were identified among the human MRSA isolates (Table 2). Eighty-five percent (6/7) of the MRSA carriers were colonized with isolates of either *spa* type t011 or t034, which are frequently associated with LA-MRSA strain ST398 [21]. One worker was colonized with a t321 strain that carried the resistance genes *drf*K and *fex*A. This strain has previously been reported to be a CA-methicillin-sensitive *S. aureus* (MSSA) belonging to MLST ST1 [22]. Regarding the LA-MRSA strains, *dfr*K was detected in both the t034 and t011 isolates, and *fex*A was found in all of the t011 isolates and 60% (3/5) of the t034 isolates. All human isolates tested negative for PVL.

A total of five environmental samples were collected from each farm, and four environmental isolates from four farms were shipped to the US laboratory. Among these isolates, three from three farms tested positive for the *mec*A gene. MRSA strains with *spa* type t321, t011, and t034 were

Table 2 Characteristics of MRSA strains isolated from swine workers in Romania.

Isolates	Farms	Sites[a]	*mec*A	*spa* type	PVL[b]	*fex*A[c]	*cfr*[d]	*dfrK*[e]	Antimicrobial resistance[f]
Human isolates									
R14	C	O	+	t321	—	+	—	+	O, T, ER
R7[h]	D	O	+	t034	—	—	—	+	O, T, CL, QD[g]
R31[h]	D	N	+	t034	—	+	—	+	O, T, CL, QD[g]
R15	D	N	+	t011	—	+	—	+	O, T, ER, CL, QD[g]
R17	E	N	+	t034	—	+	—	+	O, T, CL, QD[g]
R24[i]	F	N	+	t011	—	+	—	+	O, T
R26[i]	F	O	+	t011	—	+	—	+	O, T
R12	F	N	+	t034	—	—	—	+	O, T, ER, CL, LE, QD
R23	G	O	+	t034	—	+	—	+	O, T, ER, CL, QD
Environmental isolates									
R11	C	E	+	t321	—	—	—	—	O, T, ER, G, LE
R9	D	E	+	t011	—	—	—	—	O, T, CL, QD[g]
R19	F	E	+	t034	—	+	+	+	O, T, CL, TS, QD, LI

[a] N, nares; O, oropharynx; E, environmental.
[b] PVL, Panton–Valentine leukocidin; —, not detected.
[c] *fexA* gene; —, not detected.
[d] *cfr* gene; —, not detected.
[e] *dfrK* gene; —, not detected.
[f] O, oxacillin; T, tetracycline; ER, erythromycin; G, gentamycin; CL, clindamycin; QD, Quin/Dalfo; LE, levofloxacin; TS, TMP/SMX; LI, linezolid.
[g] Antibiotic resistant level: intermediate.
[h] Isolates R7 and R31 came from the same worker.
[i] Isolates R24 and R26 came from the same worker.

detected on farms C, D, and F, respectively. The resistance genes *fex*A, *drf*K, and *cfr* were found in the t034 strain but not in t321 or t011 strains.

Antimicrobial resistance patterns varied across the isolates. Resistance to tetracycline, oxacillin, clindamycin, and quinupristin/dalfopristin (quino/dalfo) were commonly found among the livestock-associated strains t034 and t011, and 50% of these isolates exhibited intermediate resistance to quino/dalfo. One t011 isolate and two t034 isolates also exhibited full resistance to erythromycin. Additionally, levofloxacin resistance was found in one t034 isolate. The community-associated strain t321 exhibited resistance to tetracycline, oxacillin, and erythromycin. All human isolates were susceptible to daptomycin, gentamycin, linezolid, rifampin, TMP/SMX, and vancomycin.

All three environmental isolates were resistant to oxacillin and tetracycline. Additionally, the t034 strain from farm E was linezolid-resistant, and the t321 strain from farm C was resistant to both gentamycin and levofloxacin.

Risk factors for MRSA colonization

All MRSA carriers were males who had spent fewer than 12 years in school (Table 3). Interestingly, the MRSA carriers had similar work hours, wore personal protective equipment (PPE) more often on a daily basis, and spent less time interacting with pigs compared to their MRSA-negative peers. The carriers also reported that they never brought work clothes home and always showered outside of the workplace at the end of the day (data not shown).

When asked about contact with animals outside of work, one MRSA carrier reported having been in contact with goats within the previous 12 months. None of the carriers raised pigs at home, but more than 60% of them lived with dogs. The number of pets at home appeared to be similar between the two groups, and none of the carriers had visited veterinarians recently. No significant difference was observed between the MRSA carriers and non-carriers.

Regarding general health status, most of the workers did not participate in sport activities regularly, but the majority stated that they have excellent or good health. None of the carriers reported skin diseases or hospitalizations within the previous 12 months.

Discussion

This is the first study to detect MRSA in Romanian swine workers. The results suggest that these workers are frequently colonized with diverse

Table 3 Characteristics of swine workers and MRSA carriage.[a]

Characteristic	MRSA carriage		
	Yes ($N=7$)[b]	No ($N=96$)[b]	p[c]
Demographics			
Age (years)	38.7 ± 11.6	42.4 ± 9.9	0.349
Sex			0.349
Male	7 (100)	78 (81.3)	
Female	0 (0)	18 (18.7)	
Education			0.652
<8 years	4 (57.1)	37 (38.5)	
8—12 years	3 (42.9)	45 (46.9)	
>12 years	0 (0)	14 (14.6)	
Work			
Time spent on farm (hours/week)	40.6 ± 9.9	42.5 ± 4.7	0.626
Handle pigs (hours/week)	4.3 ± 1.5	12.6 ± 6.5	<0.001
Remove pig wastes (hours/week)	6.3 ± 5.8	7.4 ± 4.1	0.632
Clean pig pens (hours/week)	8.8 ± 5.1	10.0 ± 6.8	0.693
Biosafety practice			
Daily PPE usage while working with pigs (%)			
Gloves	75.0 ± 35.4	48.9 ± 25.5	0.220
Rubber Boots	100 ± 0.0	88.9 ± 24.7	<0.001
Overalls	100 ± 0.0	96.4 ± 15.2	0.027
Contact with animals			
Number of pigs at home	0.0 ± 0.0	1.7 ± 2.0	0.234
Number of pets at home	2.5 ± 1.9	2.6 ± 2.3	0.947
Type of pets at home			0.293
Dogs	4 (66.7)	70 (75.3)	
Cats	0	5 (5.4)	
Birds	0	1 (1.1)	
Ferrets	0	1 (1.1)	
Other	1 (16.7)	1 (1.1)	
None	1 (16.7)	15 (16.1)	
Visited veterinarian within the last 12 months			0.034
Yes	0 (0)	43 (46.2)	
No	6 (100)	50 (53.8)	
General Health			
Participation in sport activities			0.373
None	5 (71.4)	76 (80.8)	
Once a week	2 (28.6)	8 (8.5)	
2—4 times a week	0 (0)	7 (7.5)	
>4 times a week	0 (0)	3 (3.2)	
Health status			0.054
Excellent	4 (57.1)	30 (31.3)	
Good	3 (42.9)	65 (67.7)	
Poor	0 (0)	1 (1.0)	
Had skin disease within the last 12 months			1.000
Yes	0 (0)	3 (3.1)	
No	7 (100)	93 (96.9)	
Had hospitalization within the last 12 months			1.000
Yes	0 (0)	4 (4.2)	
No	7 (100)	92 (95.8)	

[a] Table values are mean ± SD for continuous variables and n (column %) for categorical variables.
[b] Numbers may not sum to total due to missing data, and percentages may not sum to 100% due to rounding.
[c] p-value is for student t-test (continuous variables) or Fisher's Exact test (categorical variables).

MRSA strains that vary in genotype and antimicrobial resistance patterns. The rate of MRSA carriage among the workers (6.8%) was relatively low compared to that of other European countries (Germany: 24%, Netherlands: 42%, Spain: 9.3%) [23–25] but substantially higher than the MRSA prevalence of the general population (<0.1%) [26]; these results indicate that swine workers are a high-risk group for potential MRSA infection in Romania and may play a role in bridging the gap in MRSA transmission between pigs and humans in the community.

Our findings of two LA-MRSA *spa* types (t011 and t034) represent the first identification of LA-MRSA in Romania. Similar to the reported Romanian HA-MRSA strains [17], the majority of LA-MRSA strains in our study were resistant to tetracycline but susceptible to vancomycin. In contrast, unlike many of the reported HA-MRSA isolates, these livestock-associated strains were largely resistant to clindamycin and erythromycin and were susceptible to gentamicin and rifampin, which indicates that the forces driving antimicrobial resistance may differ between the two settings. Additionally, none of the MRSA-positive workers had recently been hospitalized. Among the human population in Romania, antibiotic use is apparently widespread. A survey conducted by the European Commission in 2010 found that more than half of the Romanians surveyed had taken antibiotics in the last year, and patients can obtain antimicrobial drugs from pharmacies without medical prescriptions [27]. Given that resistance can develop under selective antibiotic pressures [28], the widespread consumption of antimicrobials in the community may have contributed to the diverse antimicrobial resistance patterns observed in this study. The acquisition of antimicrobial resistance genes poses a major challenge to treating MRSA infections. Limiting the use of antimicrobial drugs in the community should be considered as a strategy to mitigate selective antimicrobial pressures and hamper the emergence of novel antimicrobial resistance genes.

Our findings indicate that the workers from the finishing farms (B through G) had higher rates of MRSA colonization than did the workers from the farrow-to-finish farm (A). The farm managers reported that their farms do not use antibiotics. However, a study in the United States showed that workers on antibiotic-free livestock farms can be colonized by MRSA and that the rate of colonization is comparable to that in industrial livestock operations [29]. Further investigation indicated that farm A did not import pigs from other swine facilities, farms B and C traded pigs within Romania, and farms D through G imported finishing herds from Germany, Holland, Denmark, Slovakia, and Hungary. Interestingly, *spa* types t034 and t011 have been reported among 6% and 10% of the human MRSA isolates in Germany, respectively [30]. These two *spa* types have also been detected in Denmark and the Netherlands [31], and t011 is the most frequently found strain in pig herds (50.1%) [32]. The importation of pigs may have introduced MRSA to swine farms in Romania, as a study by Broens showed that the transportation of animals is an important risk factor for the introduction of MRSA into MRSA-negative herds [33]. The swine workers may have acquired MRSA through direct transmission after handling colonized pigs. A greater number of closed production systems should be sampled in the future to examine the importation of positive-MRSA pigs in Romanian farms. This information would confirm that herd transmission of MRSA and other communicable diseases can occur during animal trading, which would make the prevention and control of infectious diseases more challenging.

In addition to interacting with pigs at their workplaces, the workers appeared to have had extensive contact with animals in their community and may have become colonized in this manner. Although none of the carriers raised pigs at home, one MRSA carrier had recently interacted with goats, which have been recognized as vectors for the zoonotic transmission of MRSA [34]. Other pets may also have acted as additional vectors, as that MRSA colonization and infection have been documented in dogs and cats [35,36]. We are unaware of any biosafety regulations regarding raising livestock at home.

We did identify a community-associated strain (*spa* t321) in both human and environmental isolates. There has been one report of MLST ST1 among pigs in Europe [37], and ST1 (PVL-negative) is prevalent among humans in Romania [38]. In addition to interacting with animals, the workers may also have become colonized after touching contaminated surfaces on the farms or vice versa, as the same MRSA isolates were found on the farms and among workers from the finishing farms C, D, and F. Positive correlations between contaminated environments and MRSA-positive pigs have been documented in other livestock facilities in Europe [11]. Because this was a pilot study, environmental samples were used for screening.

Our results likely underestimate the true prevalence of MRSA carriage on these farms. Due to the lack of the *mec*A gene, not all presumptive positive isolates detected using MRSA chromogenic agar were identified as MRSA-positive. Since the sensitivity and specificity of the chromogenic agar test are 99.5% and 97.3%, respectively, some isolates

may have been misclassified [39]. Nevertheless, this study showed that swine workers and farms in Romania often harbor LA-MRSA. Furthermore, the detection of a CA-MRSA strain (*spa* type t321) implies that swine farms are places where the exchange of human- and livestock-associated MRSA strains can occur. The distinctions between the LA-, HA-, and CA-MRSA strains have been blurred by convergent and divergent evolution, which had led to observations of isolates from different sources with the same *spa* type. Future studies, including longitudinal studies of workers and pigs that are in close proximity, are needed to elucidate the evolutionary histories of these isolates and the types of exchange that take place in commercial livestock agricultural settings.

It is possible that the mixture of small and large farms is a particular risk factor for the spread of LA-MRSA infection. Specifically, backyard livestock farming in Romania remains prevalent despite the growth of large commercial farming operations around the country; 60% of the pigs in Romania are raised in backyards or in small-scale operations for personal consumption, and 66.2% of European pigs raised on small-scale farms (<10 pigs) are found in Romania [40]. Farms in other parts of Romania may differ from those in our study. However, it is possible that in such settings, LA-MRSA can easily cross from agricultural to community environments, and future studies should explore this possibility.

This pilot study has several limitations. First is the small convenience sample. At many pig farms, there are small numbers of workers, and this is an inherent challenge to studying zoonotic transmission in swine production facilities. Additionally, this study focused on the detection of LA-MRSA among swine workers in Romania, and did not examine the transmission of MRSA from an epidemiological perspective. Sampling of pigs on farms and the animals in the workers' homes should be included in future studies, and additional closed production systems should also be considered for further investigation.

Conclusion

Our study documented the occupational risk of MRSA infection among Romanian swine workers, and our findings reinforce the need to minimize potential LA-MRSA-related infections in swine workers and their community contacts. Due to the changing landscape of livestock production in Romania, LA-MRSA is likely to persist and continue to pose health risks to livestock workers and their community contacts. Routine surveillance of MRSA infections in swine workers and pigs is recommended to better understand the epidemiology and risk factors for LA-MRSA in Romania and other countries in Eastern Europe.

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Competing interests

None declared.

Ethical approval

Not required.

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