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Nasal carriage of methicillin-resistant *Staphylococcus aureus* in cattle and farm workers in Turkey

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ABSTRACT

In the present study, nasal carriage rates of methicillin-resistant *Staphylococcus aureus* (MRSA) in cattle and humans in close contact with cattle, and the resistance rates of MRSA isolates against various antibiotics were investigated. The nasal swab samples were collected from 35 different private farms (150 humans and 250 cattle) in the Afyonkarahisar province of Turkey. The nasal carriage rates of MRSA in humans and cattle were determined as 8.7 % (13/150) and 1.2 % (3/250), respectively. MRSA strains isolated from humans and animals were not supplied from the same sampled farms. In our study, the three phenotypic tests were also compared with the PCR results. Sensitivity for oxacillin agar screening test, oxacillin and cefoxitin disc diffusion tests was found to be 75 %, 50 % and 100 %, respectively, while the specificity of the three tests was 100 %. According to the antibiotic susceptibility test results, high resistance rates were determined against clindamycin (46.1 %), kanamycin (46.1 %), mupirocin (46.1 %), erythromycin (38.5 %), fusidic acid (38.5 %) and tetracycline (38.5 %) in 13 MRSA isolates obtained from humans. Resistance to clindamycin, erythromycin, fusidic acid, mupirocin, rifampicin and teicoplanin was determined in all 3 MRSA strains isolated from cattle nasal swab samples. This is the first study to investigate the status of nasal MRSA carriage in both cattle and farm workers in contact with these cattle in Afyonkarahisar.

Key words: antimicrobial resistance, cattle, human, *mecA*, nasal MRSA carriage

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been known to be one of the most common nosocomial pathogens causing a wide range of hospital-linked infections throughout the world since the 1980s (BOYCE and CAUSEY, 1982; ENRIGHT et al., 2002; KUEHNERT et al., 2005). Since the mid-1990s MRSA has spread outside

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the hospital environment and appeared in community populations without identifiable healthcare-associated risk factors (GORAK et al., 1999; KAYABA et al., 1997; NAIMI et al., 2001). Besides its importance as a hospital and community pathogen, MRSA has also been considered as an emerging problem in veterinary medicine in recent years (SPOHR et al., 2011; VAN DUIJKEREN et al., 2010; VENGUST et al., 2006; WALTHER et al., 2008). The primary colonization site of MRSA is known as the nasal mucosa in humans and animals. Therefore, the nasal MRSA carriage has an important role in the epidemiology and pathogenesis of infections (KLUYTMANS et al., 1997; MULLIGAN et al., 1993). Various researchers have reported that it colonizes the nasal mucosa of different animal species, their owners, caretakers or veterinarians, and emphasized the importance of nasal MRSA carriage in terms of public and animal health (FAIRES et al., 2009; GORDONCILLO et al., 2012; LOEFFLER et al., 2005, VAN DUIJKEREN et al., 2010). These epidemiological studies indicate that MRSA colonizing the nasal mucosa may be transmitted between animals and humans. Accordingly, this agent has been accepted as a zoonosis and/or humanosis pathogen nowadays (FAIRES et al., 2009; MORGAN, 2008; SEGUIN et al., 1999).

Methicillin resistance is mediated by the *mecA* gene, which encodes a modified penicillin-binding protein (PBP), called PBP2a or PBP2´, with decreased affinity for almost all β-lactam antibiotics. The *mecA* locus is a highly conserved gene that encodes PBP2a in resistant strains but is absent from susceptible ones, making it a useful molecular marker of β-lactam resistance (MULLIGAN et al., 1993; PINHO et al., 2001). Also, MRSA strains are often resistant to antimicrobials other than β-lactams, of which many members are widely used in both human and veterinary medicine (LOWY, 2003; PINHO et al., 2001; RICH, 2005). Thus, the risk of treatment failure and the cost of antimicrobial therapy and hospitalization increase, while the range of therapeutic options decrease.

Several conventional methods, such as the oxacillin agar screening test, and the oxacillin and cefoxitin disc diffusion tests, have been developed to detect MRSA isolates. The reliability of these phenotypic methods has been reported to be between 80 % and 100 % (BROEKEMA et al., 2009; TIWARI et al., 2009). Alternatively, molecular techniques, mostly based on PCR amplification of the *mecA* gene, have been used for the rapid detection of MRSA (CHOI et al., 2003; KALHOR et al., 2012).

In Turkey, there are a limited number of publications on the epidemiological aspects of MRSA infections in both animals and humans; only a few veterinary reports have been published on MRSA infections in dairy herds with mastitis (CIFTCI et al., 2009; TÜRKYILMAZ et al., 2010; TURUTOGLU et al., 2009). Therefore, this study aimed to determine the prevalence of MRSA in healthy cattle and the farm workers associated with animals at different farms, and the resistance rates of MRSA isolates against various antibiotics commonly used in Turkey. This is the first study investigating the nasal

MRSA carriage in both cattle and humans in close contact with these animals in the Afyonkarahisar province of Turkey.

Materials and methods

Nasal swab samples. This study was conducted on 250 clinically healthy cattle (175 female, 75 male) and 150 volunteer humans (85 women, 65 men) in close contact with these animals on 35 different private farms in the Afyonkarahisar province of Turkey. No antibiotics had been applied to the animals in the previous one month. Also, none of the volunteers had used any antibiotic, they had not been hospitalized in the previous three months and did not have any chronic disease during the sampling period. Nasal swab samples were simultaneously taken from the both nostrils of the animals and humans using cotton swabs. Samples were stored in Stuart transport medium and were immediately transported to the laboratory in a cool box on ice.

Isolation and identification of S. aureus. Nasal swabs were inoculated into tryptone soya broth (TSB, Oxoid Ltd., Basingstoke, Hampshire, UK), supplemented with 7 % (w/v) sodium chloride, and incubated aerobically at 35 °C for 24 hours. Then, 100 µL of the broth was inoculated onto Columbia blood agar (Oxoid Ltd., Basingstoke, Hampshire, UK), containing 7 % sheep blood, and the plates were incubated under aerobic conditions for 24 hours at 35 °C (LEE et al., 2004). After the incubation, each different colony was examined macroscopically (colony morphology, haemolysis, pigment production) and microscopically (Gram staining). Identification of growing colonies was achieved using standard conventional methods. For this purpose, oxidase, catalase, slide and tube coagulase with rabbit plasma, Voges-Prouskauer, anaerobic fermentations of glucose and mannitol, and DNase activity tests were used (HOLT et al., 1994; QUINN et al., 1999). In all tests, MRSA ATCC 33591 and MSSA ATCC 25923 were used as positive and negative control strains, respectively.

Oxacillin agar screening test. To determine phenotypic resistance to methicillin, all isolates were inoculated from a direct colony suspension (0.5 McFarland standard) onto Mueller-Hinton agar (Oxoid Ltd., Basingstoke, Hampshire, UK), supplemented with 4 % (w/v) sodium chloride and oxacillin (Sigma-Aldrich Inc., St. Louis, USA) at a concentration of 6 µg/mL, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007). Oxacillin resistance was confirmed by bacterial growth after 24 hours of incubation at 35 °C on plates. Any growth was considered as a positive result for MRSA (CHAMBERS, 1997; CLSI, 2007). MRSA ATCC 33591 and MSSA ATCC 25923 were used as positive and negative control strains, respectively.

Extraction of DNA. DNA was extracted from the positive control, negative control and the test strains using a genomic DNA purification kit (MBI Fermentas, Vilnius, Lithuania) as described by the manufacturer. Briefly, one bacterial colony grown on

trypticase soy agar was inoculated into TSB and incubated at 35 °C for 18 hours. After the incubation, 1 ml was taken from the TSB and transferred to sterile DNase and RNase free 2 ml eppendorf tubes. Eppendorf tubes were centrifuged at 4000 rpm for 2 minutes. Then, the supernatant was removed and the pellet was suspended in 200 µL of sterile deionized water. The extraction was completed by following the steps as indicated in the kit.

PCR amplification conditions. For the detection of the 16S rDNA (*Staphylococcus* spp. specific) and *mecA* (methicillin resistance specific) genes, multiplex PCR was performed using the primers described by STROMMENGER et al. (2003) and CHOI et al. (2003), respectively. Five microliters of the extracted DNA were used as a template in a 25 µL PCR mixture containing 10X PCR buffer, 3 µL of 25 mM MgCl2, 0.5 µL of 10 mM dNTP mix, 0.1 µL of 20 µM each 16S rDNA and *mecA* primers, 1U of Taq DNA polymerase and deionized water. The DNAs of MRSA ATCC 33591 and MSSA ATCC 25923 were used as positive and negative controls, respectively. The PCR amplification conditions of 16S rDNA and *mecA* genes comprised an initial denaturation step at 95 °C for 5 min, and 30 cycles of 95 °C for 2 min, 54 °C for 1 min, 72 °C for 1 min and a final step 72 °C for 7 min. All products were analyzed by 1.5 % agarose gel electrophoresis and visualized using ethidium bromide under U.V. light. Molecular size markers (100-bp DNA ladder; Fermentas, Vilnius, Lithuania) were included in each agarose gel. The 420-bp and 314-bp bands were accepted as positive for 16S rDNA and *mecA* genes, respectively.

Antibiotic susceptibility test. The antibiotic resistance of MRSA strains was determined by using the Kirby-Bauer disc diffusion test on Mueller Hinton agar (Oxoid Ltd., Basingstoke, Hampshire, UK), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). For this purpose, chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), fusidic acid (10 µg), gentamicin (10 µg), kanamycin (30 µg), mupirocin (5 µg), neomycin (30 µg), rifampicin (5 µg), teicoplanin (30 µg), tetracycline (30 µg) and trimethoprim/sulfamethoxazole (25 µg) antibiotic discs (Oxoid Ltd., Basingstoke, Hampshire, UK) were used. Oxacillin (1 µg) and cefoxitin (30 µg) discs (Oxoid Ltd., Basingstoke, Hampshire, UK) were also used to compare the disc diffusion results with *mecA* PCR results. While the plates including oxacillin and cefoxitin discs were incubated at 35 °C for 24 hours, the other plates were incubated at 37 °C for 18 hours (CLSI, 2007). MRSA ATCC 33591 and MSSA ATCC 25923 were used as quality control strains.

Statistical analysis. The differences between nasal MRSA carriage and sexes in sampled humans and animals were evaluated using the Chi-square test. A probability of $P<0.05$ was considered statistically significant.

Results

Isolation and identification findings. In our study, 44 (29.3 %) and 8 (3.2 %) *S. aureus* isolates were obtained from the sampled 150 humans and 250 cattle, respectively. 16S rDNA specific bands were detected in all of 52 *S. aureus* isolates obtained from nasal samples, 16 (30.8 %) of these isolates harboured the *mecA* gene (Fig. 1). The distribution of MRSA isolates among the nasal *S. aureus* isolates was 29.5 % (13/44) for humans and 37.5 % (3/8) for cattle. These MRSA isolates were obtained from 9 out of 35 farms. The nasal carriage rates of MRSA in humans and cattle were determined as 8.7 % (13/150) and 1.2 % (3/250), respectively. The MRSA strains isolated from humans and animals were not isolated from the same farms. Also, no significant difference was determined in the human and cattle populations in terms of sexes (P>0.05). The distribution of *mecA* gene positive isolates according to sampled farm, human and cattle is shown in Table 1.

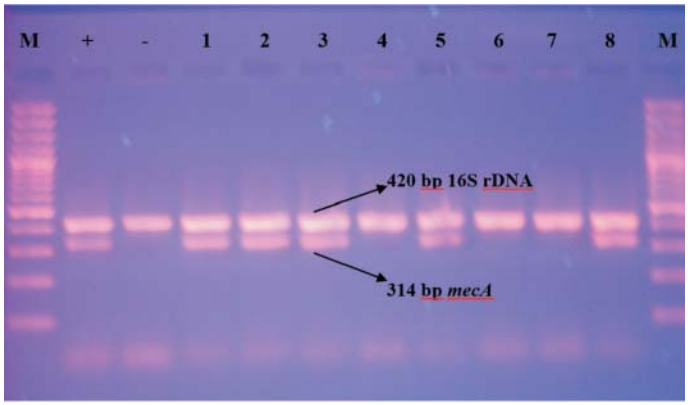

Fig. 1. 16S rDNA and *mecA* mPCR findings in *S. aureus* isolates. M: 100 bp DNA ladder (Fermentas, Vilnius, Lithuania); +: positive control (MRSA ATCC 33591); -: negative control (MSSA ATCC 25923); 1-3: *mecA* positive human isolates; 4,6,7: *mecA* negative test isolates; 5,8: *mecA* positive cattle isolates

Antibiotic susceptibility test. Twelve isolates were found phenotypically resistant to methicillin in the oxacillin agar screening test. While 8 isolates, which were susceptible to oxacillin in the disc diffusion test, were determined to be MRSA, all 16 cefoxitin resistant isolates were *mecA* positive. The comparison of the 3 phenotypic tests results with PCR is shown in Table 2.

Table 1. Number of samples and distribution of *mecA* gene positive isolates according to sampled farm, human and cattle

Private farm	No. of sample				No. of *mecA* positive isolates			
	Human		Cattle		Human		Cattle	
	Woman	Man	Female	Male	Woman	Man	Female	Male
1	4	3	15	8	-	-	1	1
2	3	2	9	6	3	1	-	-
3	2	2	9	-	-	-	-	-
4	2	1	11	6	1	-	-	-
5	2	2	7	-	-	-	-	-
6	1	1	12	6	-	-	-	-
7	2	3	4	4	-	-	-	-
8	1	3	14	2	-	-	-	-
9	1	1	1	-	-	-	-	-
10	4	4	27	9	-	-	-	-
11	2	2	1	10	1	1	-	-
12	2	1	4	1	-	-	-	-
13	3	2	4	11	-	-	-	-
14	4	3	5	-	-	-	-	-
15	3	3	5	-	-	-	-	-
16	-	1	4	-	-	-	-	-
17	2	3	6	-	-	-	-	-
18	2	2	5	-	-	-	-	-
19	2	-	4	2	-	-	-	-
20	2	-	1	-	-	-	-	-
21	3	1	4	-	1	-	-	-
22	1	1	4	1	-	-	-	-
23	2	2	5	-	-	-	-	-
24	2	2	2	-	-	-	-	-
25	3	3	2	-	1	-	-	-
26	1	1	1	-	-	-	-	-
27	4	-	2	-	-	-	-	-
28	3	2	1	1	-	-	-	-
29	2	1	-	2	-	-	-	-
30	1	1	1	-	-	-	1	-
31	1	3	-	4	-	-	-	-
32	1	1	2	-	-	-	-	-
33	10	3	-	2	3	-	-	-
34	2	2	1	-	-	1	-	-
35	5	3	2	-	-	-	-	-
Total	85	65	175	75	10	3	2	1

Table 2. Comparison of three phenotypic tests with *mecA* PCR

PCR *mecA*	No. of isolates	OAS		ODD		CDD	
		R (%)	S (%)	R (%)	S (%)	R (%)	S (%)
Positive	16	12 (75)[a]	4 (25)	8 (50)[a]	8 (50)	16 (100)[a]	0
Negative	36	0	36 (100)[b]	0	36 (100)[b]	0	36 (100)[b]

*OAS: Oxacillin agar screening, ODD: Oxacillin disc diffusion, CDD: Cefoxitin disc diffusion, R: Number of resistant isolates, S: Number of sensitive isolates, a: Sensitivity, b: Specificity

According to disc diffusion test results, high resistance rates were determined against clindamycin (46.1 %), kanamycin (46.1 %), mupirocin (46.1 %), erythromycin (38.5 %), fusidic acid (38.5 %) and tetracycline (38.5 %) in 13 MRSA isolates obtained from humans. Also, in all 3 MRSA strains isolated from cattle nasal swab samples, the resistance to clindamycin, erythromycin, fusidic acid, mupirocin, rifampicin and teicoplanin was determined. The antibiotic resistance of MRSA isolates is shown in Table 3.

Table 3. Antibiotic resistance of 16 MRSA isolates obtained from nasal samples

Antibiotic	Farm workers (n = 13) resistance		Cattle (n = 3) resistance	
	n	%	n	%
Chloramphenicol	-	0	1	33.3
Ciprofloxacin	2	15.4	-	0
Clindamycin	6	46.1	3	100
Erythromycin	5	38.5	3	100
Fusidic acid	5	38.5	3	100
Gentamicin	4	30.8	-	0
Kanamycin	6	46.1	-	0
Mupirocin	6	46.1	3	100
Neomycin	2	15.4	1	33.3
Rifampicin	3	23.1	3	100
Teicoplanin	4	30.8	3	100
Tetracycline	5	38.5	-	0
Trimethoprim/sulfamethoxazole	1	7.7	-	0

Discussion

This study investigated the nasal carriage of MRSA in cattle and farm workers in contact with these cattle, and the resistance of MRSA isolates to various antibiotics commonly used in Turkey.

The presence of MRSA in animals and humans in the many countries is of veterinary and public health concern. Nasal carriage has an important role in the epidemiology and pathogenesis of MRSA infection in humans and animals (KLUYTMANS et al., 1997; MULLIGAN et al., 1993). Several reports show the prevalence of MRSA in cattle with mastitis (CIFTCI et al., 2009; SPOHR et al., 2011; TÜRKYILMAZ et al., 2010; TURUTOGLU et al., 2009), but compared with other animals, there is much less information about the nasal colonization of MRSA in cattle (ALZOHAIRY, 2011; ERDEM and TÜRKYILMAZ, 2013; HUBER et al., 2010; İNEGÖL and TÜRKYILMAZ, 2012). ALZOHAIRY (2011) investigating the nasal carriage in different farm animals reported that the nasal MRSA carriage rate was 15.5 % in cattle. In a study made in farm animals, the nasal MRSA prevalence was found to be 0.3 % and 1 % in 400 cattle and 300 calves, respectively (HUBER et al., 2010). In Turkey, ERDEM and TÜRKYILMAZ (2013) investigated the presence of MRSA in cows and farm workers and reported that 4 of 56 bovine and 6 of 34 human nasal isolates were positive for MRSA. In another study from Turkey, methicillin resistance was detected in 26.8 % (15/56) and 62.8 % (22/35) of bovine nasal and human nasal isolates, respectively (İNEGÖL and TÜRKYILMAZ, 2012). In our study, the nasal carriage rates of MRSA in 150 humans and 250 cattle from 35 different farms were determined as 8.7 % (n = 13) and 1.2 % (n = 3), respectively. The prevalence found for both cattle and farm workers in the current study was lower than in other two studies from Turkey. The difference between the sample size and geographical variations may be the causes of this discrepancy. MRSA strains isolated from humans and animals in our study were not isolated from the same farms, and this finding was remarkable for us. We considered this may indicate that cattle are not the source of MRSA for the investigated farm workers.

Several phenotypic methods have been preferred by various researchers in the detection of methicillin resistance. Generally, oxacillin agar screening (OAS) test has been commonly used among these tests. Similar opinions have been reported on the specificity and sensitivity of the OAS test (KALHOR et al., 2012; TIWARI et al., 2009). In MRSA strains, TIWARI et al. (2009) found the sensitivity and specificity of the OAS test to be 87.9 % and 94.9 %, respectively. In another study, the sensitivity and specificity of the phenotypic agar screen method for determination of MRSA strains were found to be 81.5 % and 96 %, respectively (KALHOR et al., 2012). We compared the OAS results with *mecA* PCR in the present study. According to our findings, while the specificity of OAS test was 100 %, the sensitivity of this test was determined as 75 %. This difference may be due to diversity in strains, the number of isolates and origin of samples.

CLSI recommends usage of cefoxitin instead of oxacillin when using the disk diffusion method to determine resistance against methicillin for *S. aureus* (CLSI, 2007). Similarly, various researchers reported that cefoxitin results were easier to interpret and were thus more sensitive for the detection of *mecA*-mediated resistance than oxacillin results (BROEKEMA et al., 2009; CAUWELIER et al., 2004; TIWARI et al., 2009). In a study,

while the sensitivity was 83.5 % for oxacillin and 91.7 % for cefoxitin discs, the specificity of these discs was determined as 100 % (CAUWELIER et al., 2004). BROEKEMA et al. (2009) reported the sensitivity of 97.3 % and specificity of 100 % for the cefoxitin disc. In another study, the sensitivity and specificity rates were found to be 77.3 % and 84.6 % for oxacillin and 98.5 % and 100 % for cefoxitin in MRSA strains (TIWARI et al., 2009). Compared with other studies, in our study, the relatively poor sensitivity (50 %) was seen in the oxacillin disc diffusion test. However, the sensitivity of cefoxitin tests was found to be 100 %, while the specificity for the two tests was 100 %. According to these findings, we considered that the cefoxitin disc may be used alone and reliably with PCR in the routine diagnosis of MRSA strains.

MRSA strains have been recognized to be resistant to almost all β-lactam antibiotics (LOWY, 2003; PINHO et al., 2001). We investigated the antibiotic resistance of MRSA strains isolated from human and cattle nasal swab samples against various antibiotics commonly used in Turkey. According to disc diffusion test results, high resistance rates were determined against clindamycin (46.1 %), kanamycin (46.1 %), mupirocin (46.1 %), erythromycin (38.5 %), fusidic acid (38.5 %) and tetracycline (38.5 %) in 13 MRSA isolates obtained from humans. The resistance to mupirocin and fusidic acid, recommended for the treatment of MRSA infections, was a prominent finding for us. The reasons of this result may be intensive, uncontrolled and prolonged use of and accessibility to these antibiotics without a medical prescription (ÇAKIR, 2001). In the present study, the resistance was also found to clindamycin, erythromycin, fusidic acid, mupirocin, rifampicin and teicoplanin in all 3 MRSA isolates isolated from cattle nasal samples. These very high resistance rates may be associated with the low isolate number of isolates.

In conclusion, we have reported the first isolation of MRSA from cattle and cattle workers in the Afyonkarahisar province of Turkey. Although the extent of this problem in the Turkey is currently unknown, our findings may have important implications for the nasal carriage status of MRSA. The resistance rates to mupirocin and fusidic acid, suggested for treatment of MRSA infection in humans, were noteworthy. Our study may indicate that cattle are not a source of MRSA for the investigated farm workers. Nevertheless, there is a need for awareness, appropriate intervention and control measures to prevent the spread of MRSA between animal and human populations. Also, the data deficiency on the MRSA epidemiology of humans and animals populations in Turkey should be eliminated throughwith future investigations.

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SAŽETAK

Istražene su stope kliconoštva izolata bakterije *Staphylococcus aureus* otpornih na meticilin (MRSA) u goveda i ljudi koji su bili u izravnom dodiru s njima te otpornost MRSA izolata na različite antibiotike. Uzorci nosnog obriska bili su prikupljeni na 35 privatnih gospodarstava (150 uzoraka od ljudi i 250 od goveda) na području Afyonkarahisara u Turskoj. Stopa nosnog kliconoštva MRSA u ljudi bila je 8,7 % (13/150), a u goveda 1,2 % (3/250). MRSA izolati iz ljudi i životinja nisu potjecali iz istih farmi. Rezultati pretrage trima fenotipskim postupcima bili su uspoređeni s rezultatima pretrage PCR-om. Stopa osjetljivosti pretraženih izolata u probirnom testu na oksacilin iznosila je 75 %, a u disk difuzijskom testu na oksacilin 50 % te na cefoksitin 100 %. Specifičnost svih triju testova bila je 100 %. Velika stopa otpornosti u 13 MRSA izolata iz ljudi ustanovljena je na klindamicin (46,1 %), kanamicin (46,1 %), mupirocin (46,1 %), eritromicin (38,5 %), fuzidinsku kiselinu (38,5 %) i tetraciklin (38,5 %). Otpornost na klindamicin, eritromicin, fuzidinsku kiselinu, mupirocin, rifampicin i teikoplanin dokazana je u tri MRSA izolata iz uzoraka nosnog obriska goveda. Ovo je prvo istraživanje nosnog kliconoštva MRSA u goveda i radnika, koji su bili u dodiru s govedima na farmama u Afyonkarahisaru.

Ključne riječi: otpornost na antibiotike, govedo, čovjek, *mecA*, nosni obrisak, MRSA, kliconoštvo