Detection of methicillin-resistant *Staphylococcus aureus* in broiler and broilers farm works in Duhok, Iraq by using conventional and PCR techniques

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Abstract

Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) has become a global public health concern. The purpose of this study was to investigate the prevalence rates of MRSA infection amongst broiler chickens and broilers farm workers. The total samples used in this study were 306. Cloacal swab samples from 231 broilers and nasal swab samples from 75 broilers farm workers were collected from five farms in Duhok city, Iraq. Isolation and identification of MRSA isolates were carried out and the antibiotic susceptibility were screened. Molecular characterization of all isolates was performed by using polymerase chain reaction (PCR) technique to detect the *mec*A gene. *S. aureus* was detected among 84% (63/75) of the farms workers samples and among 84.8% (196/231) of the broiler's samples. The *S. aureus* isolated from farm workers and broilers appeared resistant to oxacillin 28.6% (18/63), and 32.1% (63/196), respectively. MRSA colonization in farm workers and broilers was 24% (18/75) and 27.3% (63/231) respectively. The *S. aureus* isolates showed the most resistant to chloramphenicol and the least resistant to vancomycin. The results of the PCR assays revealed that 85.7% (12/14) of *S. aureus* isolates from farm workers and 44.4% (16/36) of *S. aureus* isolates from broilers were positive for the *mecA* gene. The direct handling of broilers by farm workers plays the important role for transport the MRSA isolates from broilers to broilers farm workers.

Keywords: MRSA, *S. aureus*; *mec*A gene; Broilers; Farm workers
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تحديد المكورات العنقودية الذهبية المقاومة للمثيسيلين في الدواجن وعمال حقول الدواجن في دهوك ـ العراق باستخدام الطرق التقليدية و تفاعل البلمرة المتسلسل

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الخلاصة

أصبحت جراثيم المكورات العنقودية المقاومة للمثيسيلين MRSA المصاحبة للدواجن مقلقة للصحة العالمية العامة. كان الغرض من هذه الدراسة هو التحقق في مدى انتشار معدلات الاصابة بجرثومة MRSA بين الدواجن وعمال حقول الدواجن. تم جمع ٣٠٦ عينة. تم جمع ٢٣١ عينة من مسحات المخرج للدجاج و ٧٥ عينة من مسحات الانف لعمال الدواجن من خمسة حقول للدواجن في مدينة دهوك، العراق. تم اجراء عزل وتشخيص سلالات MRSA وتم اجراء فحص حساسيتها للمضادات الحيوية. تم إجراء التوصيف الجزيئي لجميع العزلات بواسطة تفاعل البلمرة المتسلسل للكشف عن الجين. *mecA*تم تحديد جراثيم المكورات العنقودية الذهبية في ٨٤٪ (٧٥/٦٣) من عينات

عمال الحقول و٨٤,٨٪ (٢٣١/١٩٦) من عينات دجاج اللحوم. أظهرت عزلات المكورات العنقودية المعزولة من الدجاج وعمال الحقول نسبة مقاومة للمضاد الحيوي الاوكزاسلين ٣٢,١% (١٩٦/٦٣) و ٢٨,٦% (٨٣/١٨) على التوالي. كان تواجد MRSA في عمال المزارع والدجاج ٢٤ ٪ (٧٥/١٨) و ٢٧,٣ ٪ (٢٣١/٦٣) على التوالي. كانت عزلات جراثيم المكورات العنقودية من الانسان والدجاج ذات مقاومة عالية للكلورامفينيكول وذات مقاومة قليلة بالنسبة للفانكومايسين. أظهرت نتائج تفاعل البوليميرز المتسلسل أن ٨٥,٧% (١٤/١٢) من عزلات المكورات العنقودية المعزولة من عمال الحقول و ٤٤% (٣٦/١٦) من عزلات الدجاج كانت موجبة بالنسبة للجين *mecA*. قد يلعب التلامس المباشر للدجاج من قبل عمال الحقول دورا هاما في انتقال جراثيم المارسا من الدجاج الى عمال الحقول.

Introduction

Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen capable of causing severe disease to farm workers and domesticated animals (1). *S. aureus* has ability to acquire resistance to several types of antibiotics. In the early 1940s, the concept of resistance of *S. aureus* isolates to antibiotic therapy was raised, and the prevalence of the antibiotic resistance has dramatically increased in recent decades due to misuse used of antibiotics and the prescription that, used for therapeutics of diseases (2). Since 1959, penicillin-resistant *S. aureus* infections have been successfully treated with methicillin. However, in 1961, there were several of the reports from the United Kingdom showed that *S. aureus* isolates had appeared acquired resistance to the methicillin and this was first identification of the methicillin-resistant *S. aureus* strains (3).

The MRSA infection was first subdivided into hospital-associated (HA-MRSA) and community-associated MRSA (CA-MRSA) infections. In addition, a third group has been emerged and known as livestock-associated MRSA (LA-MRSA) (4-6). *S. aureus* isolated from poultry has become a serious zoonotic risk factor on a global scale for the farm workers groups who handle or live in close proximity to chickens (1). The infection with LA-MRSA has been globally documented (2, 3). At present, MRSA strains have been identified by using the biochemical test to detect the phenotypical characterization of MRSA which appeared resistance to all the antibiotics that have been manufactured to date (4, 5). The *mec*A gene, a (associated with resistance in MRSA) encodes the altered penicillin binding protein (PBPa), which has a low affinity for β-lactam group antibiotics and prevent it to link with β-lactams. The aims of this study were to investigate the prevalence of *S. aureus* and MRSA among broiler chickens and broilers farm workers. In addition, to assess the presence of the *mec*A gene in MRSA isolates using PCR in different farms in Duhok governorate, Iraq.

Materials and methods

Study design and sampling

A total of 306 swab samples were collected, 231 samples from broiler chickens and 75 samples from farm workers were included (Table 2). The study was conducted from December 2016 to October 2017. The study was based on voluntary participation. The samples were collected in a randomly selected from 12 rural poultry farms from all districts in Duhok city. The farms subdivided into five groups according to their location in different districts in Duhok governorate (Akre, Amedi, Bardarash,shekan and Zakho) (Table 2). The farm workers nasal samples were obtained by inserting a sterile swab 2 cm into the nostril and gently rotated against the mucosal surface for approximately 5-10 second. The broilers samples were obtained by inserting a sterile swab stick in the cloaca and gently rubbing, the swab against the mucosal surface for approximately 5-10 second. The farm workers and broilers samples were transport into 10 ml trypticase soy broth containing 70 mg/ml NaCl and incubated at 37ºC for 24 hrs.

Cultural, biochemical and molecular identification of *S. aureus* and MRSA

A loopful of the inoculum from the enrichment broth was streaked on the mannitol-salt agar and incubated at 37ºC for 24 hrs. The suspected *S. aureus* colonies were selected and sub-cultured on the mannitol-salt agar to obtain a pure culture. The presumptive colonies of *S. aureus* were further identified microscopically and biochemical tests. The strains were considered as *S. aureus* relying on mannitol salt agar fermentation, gram stain, morphology, catalase test and coagulase test. MRSA isolates were identified upon their resistance to an oxacillin 6 µl/ml using agar screening method (6). MRSA isolates were further identified based on the presence of the *mec*A gene using PCR-based screening method.

Extraction of chromosomal DNA and polymerase chain reaction (PCR)

The DNA of the *S. aureus* isolates was extracted by using commercial DNA isolation kit according to manufacturer instructions (AccuVis Bio, UAE). The purity and concentration of DNA of each isolate was measured by using Nanodrop device (Thermo scientific-USA). The PCR technique was used to screen the existence of the *mec*A gene in MRSA isolates utilising two previously described primers, MR1 forward (5'GTGGAATTGGCCAATACAG G3') and MR2 reverse primer (5'TGAGTTCTGCAGTACC

GGAT3') (7, 8). The reactions were achieved using a PCR Thermocycler (Applied biosystem, USA). Reactions were performed in a 20 µl final volume containing 10 µl of master mix, 2 µl *S. aureus* genomic DNA (final concentration 50-100 ng/µl), 1 µl of each primer MR1 and MR2 at final concentration 5 µM, and 6 µl of distilled water. Amplification conditions were: denaturation at 95°C for the 30s, annealing at 55°C for 1 min, extension at 72°C, denaturation for 1 min at 95 °C, (for 33 cycles), and a final extension for 5 min at 72°C (9). PCR products (bands) were visualized after gel electrophoresis at 100 V in a 1.5% agarose gels using UV Transilluminator (Cleaver scientific, UK).

Antibiotic sensitivity test

The agar screening method was used to investigate the antibiotic susceptibility according to the Clinical Laboratory Standards Institute (CLSI) recommendations (6). The nine different antibiotics from different antimicrobial groups were used in this study that supplied by Bioanalyse (Turkey) (Table 1). A single colony was selected from an overnight culture and inoculated into 5 ml of brain heart infusion broth and then incubated at 37°C overnight. The desired antibiotic with final concentration was prepared on Muller Hinton agar or brain heart infusion (BHI) agar (Oxoid, England). Bacterial suspension was tested to obtain a density of 0.5 McFarland turbidity then 10 µL drop was spotted onto agar plate surface and were incubated at 35°C overnight. The vancomycin resistance was defined as the capability of growth in BHI agar screening media (6 µg/ml vancomycin). Growth was considered as a positive result and the isolates were further investigated (MIC ≥16 µg/ml) to confirm the identifications according to CLSI recommendations.

Ethics statement

Informed written consent was obtained from farm workers. The study was conducted with the approval of ethics committee in the University of Zakho, Faculty of Sciences, Iraq.

Data analysis

Chi-squared test was used to assess the associations between variables. All statistical analysis was performed using the SPSS 18 software. P<0.05 were considered as significant.

Table 1: Antimicrobial agents used in the current study

Antibiotic group	Antibiotic	µg/ml
Beta-Lactams (Penicillins)	Oxacillin (OXA)	6
Glycopeptides	Vancomycin (VAN)	6
Phenicoles	Chloramphenicol (CHL)	32
Quinolones	Ciprofloxacin (CIP)	4
Macrolides	Tilmicosin (TIL)	8
Tetracyclines	Doxycyclin (Dox)	16
Aminoglycosides	Amikacin (AMK)	64
Beta-Lactams (Carbapenems)	Meropenem (MEM)	16
Macrolides	Erythromycin (ERY)	8

Results

Prevalence of *S. aureus* and MRSA in farm workers and broilers in different farms

In the current study, the carriage rate of *S. aureus* among broilers in all farms was 84.8% (196/231) whereas it was 84% (63/75) among farm workers. The differences of the incidence rates of *S. aureus* in broilers and broilers farm workers in all farms was statistically non-significant (p= 0.85). However, in broilers, there were significant differences of the prevalence of *S. aureus* in different farms (p= 0.0001) and the prevalence ranged from 60% to 97% (Table 2).

The *S. aureus* nasal carriage rate among farm workers in different farms was significantly different (p= 0.02). When comparing individual farms, MRSA infection prevalence was as high 100% to as low as 46% amongst farm workers (Table 2).

Table 2: Distribution of *S. aureus* and MRSA isolates among broilers and broilers farm workers in different farms

Farm groups	*S. aureus*				MRSA			
	Broilers	*P* value	Farm workers	*P* value	Broilers	*P* value	Farm workers	*P* value
Akre	42/46 (91.3%)	0.0001	21/21 (100%)	0.02	6/46 (13%)	0.001	1/21 (4.8%)	0.0003
Amediya	49/53 (92.5%)		7/13 (53.8%)		19/53 (35.8%)		1/13 (7.7%)	
Bardarash	18/30 (60%)		8/8 (100%)		3/30 (10%)		6/8 (75%)	
Shekan	45/59 (76.3%)		15/17(88.2%)		25/59 (42.4%)		7/17 (41.2%)	
Zakho	42/43 (97.7%)		12/16 (75%)		10/43(23.3%)		3/16 (18.8%)	
Total	196/231(84.8%)		63/75 (84%)	0.85	63/231(27.3%)		18/75 (24%)	0.57

S. aureus isolates from farm workers groups and broilers from all farms were screened for resistance to oxacillin. Amongst farm workers groups, 24% (18/75) of the *S. aureus* isolates where identified as MRSA carrier. While in broilers, 27.3% (63/231) were colonized with MRSA (Table 2). There was a significant difference between the distribution of MRSA among farm workers (p= 0.0003) or broilers (p= 0.001) amongst all farms. However, when comparing resistance rate of MRSA isolates in all farms, the occurrence of MRSA was statistically non-significant (p= 0.57).

In broilers, the incidence rate of MRSA isolates among farms was statistically different and the carriage rates ranged from 13% to 42.4%. The carriage rate of MRSA isolates among farm workers was significantly different in different farms (ranged from 5% to 75%). The highest rate (42.4%) of MRSA among broilers was found in Shekan farms while the highest rate (75%) in farm workers was observed in Bardarash farms.

Antibiotic susceptibility of *S. aureus* and MRSA in farm workers and broilers in different farms

The susceptibility of MRSA isolates from farm workers and broilers against different antibiotics from different farms were variable. Generally, farm workers MRSA isolates showed different patterns of resistance toward antibiotics in comparison to broilers isolates. Amongst the 18 farm workers MRSA isolates. The highest resistant rate of *S. aureus* isolates was against chloramphenicol was 83% (15/18). Moreover, farm workers MRSA resistance was 39% against dyoxycillin, 28% to ciprofloxacin, 22% to amikancin, 11% to tilmicosin and meropenem, 5.5% to erythromycin, and 5.5% to vancomycin. While amongst broilers MRSA, the highest resistant percentage was observed against chloramphenicol 81% (51/63) followed by ciprofloxacin and dyoxycillin which were 63% and 49%, respectively. The resistance of MRSA isolates toward meropenem was 57%, 43% to amikancin, 33% to tilmicosin, 22% to erythromycin and 3% to vancomycin. The resistance of broilers and farm workers MRSA isolates was significantly different against ciprofloxacin and meropenem (*P* value was 0.023 and 0.003 respectively) (Table 3).

Molecular detection of the *mecA* gene in MRSA isolates

The MRSA isolates were identified based on their phenotypic resistance to oxacillin. However, the antibiogram typing (oxacillin-agar screen test) is not able to confirm the presence of the *mec*A gene. Therefore, PCR was used to screen the existence of the *mec*A gene in MRSA isolates.

Molecular identification of MRSA strains was performed by detection of the *mecA* gene in MRSA strains using PCR. 50 MRSA isolates (36 from broilers and 14 from farm workers) from different farms were selected and screened for the *mec*A gene. The amplified PCR products for the identification of the *mec*A gene were visualized on the agarose gel and the expected band was observed (1,339 bp) (Figure 1). 56% (28/50) of the MRSA isolates were positive for the *mec*A gene. The existence of the *mec*A gene among farm workers and broilers MRSA isolates was statistically significant different (p= 0.008). Amongst the farm workers MRSA, 85.7% (12/14) isolates were *mec*A-positive. While, 44.4% (16/36) of the broiler's MRSA isolates were *mec*A-positive.

Table 3: Antimicrobial resistance patterns of the MRSA isolates from farm workers and broilers in different farms

Farms	Source	*S. aureus*	Antimicrobial agents								
			OXA R (%)	VAN R (%)	CHL R (%)	CIP R (%)	Dox R (%)	AMK R (%)	MEM R (%)	ERY R (%)	TIL R (%)
Akre	W	21	1 (5)	0(0)	0 (0)	1(100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	B	42	6 (14)	0 (0)	4 (67)	6 (100)	0 (0)	0 (0)	1(16)	4 (67)	3 (50)
Amediya	W	7	1 (14)	0 (0)	0 (0)	0 (0)	1 (100)	1(100)	0 (0)	0 (0)	0 (0)
	B	49	19 (38)	1 (5)	17 (90)	17 (90)	15 (79)	9 (47)	12 (62)	4 (21)	9 (47)
Bardarsh	W	8	6 (75)	0 (0)	6 (100)	3 (50)	0 (0)	0 (0)	1 (16)	1(16)	1(16)
	B	18	3 (17)	0 (0)	1 (33)	3 (100)	0 (0)	0 (0)	0 (0)	2 (66)	1 (33)
Shekan	W	15	7 (46)	0 (0)	4 (58)	0 (0)	5 (71)	3 (42)	2 (28)	0 (0)	1 (14)
	B	45	25 (56)	1 (4)	25(100)	13 (70)	14 (43)	16 (88)	22(100)	4 (23)	4 (18)
Zakho	W	12	3 (25)	0 (0)	1 (33)	1 (33)	1 (33)	0 (0)	0 (0)	0 (0)	0 (0)
	B	42	10 (23)	0 (0)	4 (40)	1 (10)	2 (25)	0 (0)	1(10)	0 (0)	4 (40)
Total	W	63	18(28.6)	1 (5.5)	15 (83)	5 (28)	7 (39)	4 (22)	2 (11)	1 5.5)	2(11)
	B	196	63(32.1)	2 (3)	51 (81)	40 (63)	31(49)	27(43)	36(57)	14 (22)	21(33)
P value			0.594	0.711	0.726	0.023	0.358	0.114	0.003	0.100	0.067

* W= Workers, B= Broilers.

Discussion

Staphylococci spp. can cause different patterns of infections in farm workers and broilers. About 20% of the population are almost permanently colonized and 60% are intermittent carriers, whereas 20% has never carried *S. aureus* (10). In the current study, similar rate of *S. aureus* occurrence was found in broilers and farm workers (84.8% and 84% respectively). However, among broilers, there were significant differences of the prevalence of *S. aureus* in different farms. Also, there was significant difference in carriage rate of *S. aureus* among farm workers in different farms. Studies showed different rates of *S. aureus* in different regions (11, 12). Our study showed higher rate compared to a study performed in Mosul-Iraq by Shareef *et al*., (13), who reported an incidence rate of *S. aureus* in poultry, ranging from 62.5 to 79.16%. Also, Bakeet and Darwish (14) reported the rate 72.5% of *S. aureus* isolates from chickens samples in Egypt. However, the *S. aureus* rate in our study was lower than the rate that detected by Thompson *et al*., (15) who reported a high rate (97.9%) of *S. aureus* occurrence among chickens.

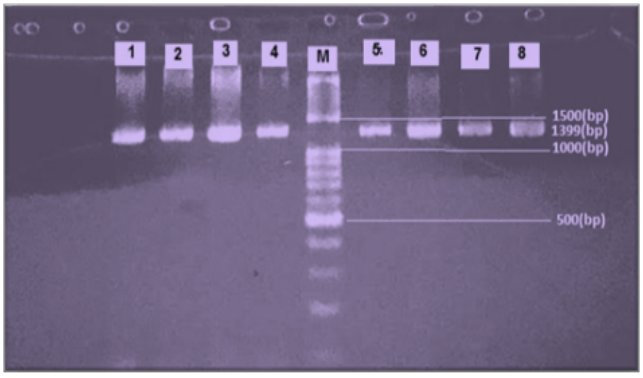

Figure 1: The amplified PCR-products for the *mec*A gene from MRSA isolates using MR1 and MR2 primers. M: DNA ladder. Lanes 1,2,3, and 4 represent MRSA isolates from farm workers. Lanes 5,6,7, and 8 MRSA isolates from broilers.

The prevalence of *S. aureus* nasal carriage among healthy adults is highly variable, with strong associations with high farm workers population density (16). Studies showed that the carriage rates of *S. aureus* among university students, secondary school student, and healthcare staff were 17.5%, 18.4% and 22.5%, respectively (17-19). The high prevalence of *S. aureus* among farm workers in present study may be due to the regular direct contact with large number of poultry on each farm (ranging between 20000 broilers per farm). Also, the high prevalence of *S. aureus* among farm workers could be due to carrying and handling broilers for treatment purpose and inadequate cleaning of farm facilities contaminated with chicken feces. In an epidemiological study reported the presence of *S. aureus* in 50% of dust samples obtained from poultry farms environments (20). Contamination of the farm worker's food or water supplies with chicken fecal or food residues may be one route by which MRSA transmission from these animals to farm workers groups may be taking place (21).

Amongst farm workers groups, MRSA carrier was 24%, while in broilers it was 27.3%. Moreover, the incidence rate of MRSA isolates among broilers in different farms was significant. Also, it was significant among farm workers in different farms. Studies showed various rates of the distribution of MRSA among farm workers and chickens (22, 23). MRSA prevalence rates were lower than that reported by Richter *et al*., (24) which found that the prevalence rate of MRSA was (71.5%) and Suk-kyung *et al.* (25) was (43.3%). Furthermore, the MRSA-positive broilers rate in this study was higher than the finding of other studies which showed a low prevalence of MRSA among broilers (22, 24, 26, 27). In contrast, Geenen *et al.* (28) found that MRSA strains amongst people living and/or working in these farms and chickens were only 5.5% and 8% respectively.

The high prevalence of MRSA among farm workers groups may be attributed to prolonged physical contact of people and broilers (especially when farm workers treat, vaccinate, and clean livestock) (29). The prevalence of MRSA among broilers is probably due to the high and unrestricted uses of antimicrobial drugs on large farms. The use of most antimicrobial agents on poultry farms, food supplements, and prophylaxis has been reported to be a hazard and risk factor for the prevalence and spread of MRSA infection (30). Antimicrobial drug misuse therefore is a major factor for the spread of MRSA from domesticated animals to farm workers.

In the current study, 32.1% of broilers MRSA isolates and 28.6% of farm workers MRSA isolates were resistant to oxacillin. Results from other studies showed varied resistance of MRSA isolates towards different antibiotics (31, 32). Our results partially come in accordance with Mulders *et al.,* (33) findings who recorded that the antibiotic resistance was 30% for erythromycin and 42% for ciprofloxacin. However, Neela *et al.* (22) recorded that all isolates were resistant to ciprofloxacin.

In the current study one MRSA isolate from farm workers (5.5%) and two from broilers (3%) were resistance toward vancomycin. Several studies showed that MRSA isolates were resistant to vancomycin (34, 35). While other investigations showed that all MRSA isolates were susceptible to vancomycin (22, 36). The high resistant to vancomycin in poultry may be due the use of macrolide antibiotics such as tylosin as growth promoter and prophylaxes which can be linked to vancomycin-resistance

genes *(van*A). The relationship of vancomycin resistance to macrolide resistance has also been detected in farm workers groups (37). Vancomycin is regarded to be a drug of choice for farm workers MRSA infections, but it is possible that the occurrence and spread of new vancomycin-resistant strains from animal source might therefore possibly have very serious consequences for the treatment of MRSA infections in farm workers groups. In addition, although some antibiotics are only used in veterinary medicine, such as tilmicosin, may spread vancomycin resistant MRSA to groups. Furhtermore, presence of high levels of antibiotic resistance in *S. aureus* and MRSA in chickens destined for the food supply chain are worrying because such strains could be passed to farm workers through the consumption of poultry (38).

Recognition of the *mec*A gene is regarded as the gold standard for the diagnosis of MRSA isolates (39). However, studies showed varied rates of the *mec*A gene existing among MRSA isolates from poultry farm workers and among healthy individuals (i.e. CA- MRSA) (19, 36).

It is found that amongst the tested farm workers MRSA isolates, 85.7% were *mec*A-positive. While, 44.4% of the tested broilers MRSA isolates were *mec*A-positive. The lack of the *mec*A gene within MRSA isolates in both farm workers and broilers have been widely reported. The current result is similar to observations by Lee (41) who reported that only 53% MRSA isolates were *mec*A positive in broilers. However, our results was higher than Mulders *et al.*, (38) who found only 28% poultry MRSA were carrying the *mec*A gene. A study from Egypt on broiler chickens MRSA infections prevalence revealed that all isolates were carrying the *mec*A gene (14). But, in a study in Jordan, 71% of MRSA isolated from chickens found to be *mec*A positive (34). Additionally, Nemati *et al.* (40) reported that 81% of chickens *S. aureus* isolates carried the *mec*A gene.

The absence of the *mec*A gene within resistant MRSA isolates may be due to hyper-production of β-lactamase by the bacteria (41), or due to particular modifications in different amino acids existing in penicillin binding proteins cascade (PBPs 1-3), which may be the origin of resistance (42). These support the idea that there are specific mechanisms or alternative genes rather than the existence of the *mec*A gene which contributes for beta-lactam resistance of MRSA.

Conclusion

High prevalence of *S. aureus* and MRSA were observed in both farm workers and chickens. All MRSA isolates in farm workers and broiler chickens were highly resistant to chloramphenicol. Resistant to vancomycin antibiotics was observed. All MRSA isolates from both farm workers and broilers showed multi-drug resistant patterns. Additionally, not all MRSA strains possessed the *mec*A gene. Hence, this should be taken into consideration by regional and reference laboratories. Further investigations are needed to analysis the molecular epidemiological relatedness of MRSA isolates from farm workers and poultry.

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