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Research Article

Antibiotic Resistance Gene Profiles of *Staphylococcus aureus* Isolated From Foods of Animal Origin [1]

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Abstract

In this study, the investigation of the antibiotic resistance gene profiles of *Staphylococcus aureus* isolates from foods of animal origin was aimed. Totally, 95 *S. aureus* strains, obtained during a period between 2009 and 2012, from culture collection of the Food Hygiene and Technology Laboratory, were examined. The isolates were confirmed by phenotypic tests and PCR. The antibiotic susceptibilities of the isolates were analyzed by disc diffusion method and the minimal inhibition concentrations of the antibiotics were determined by E test. PCR were also utilized for determining the presence of resistance genes including *bla*Z, *erm*A, *erm*C, *tet*K, *tet*M, *mec*A, *Van*A, *Van*B, *Vat*A, *Vat*B and *aacA-aphD*. Resistance to penicillin, tetracycline, vancomycin, erythromycin, cefoxitin, gentamycin and quinupristin-dalfopristin were evident as 81.1%, 28.4%, 18.9%, 17.9%, 9.4%, 9.4% and 3.2% respectively. E test results were compatible with the disc diffusion method. Multidrug resistance was observed from 29.5% of *S. aureus* isolates. Positive compatibility was observed between conventional methods and PCR for the resistance of the isolates, except for vancomycin. In addition, all of the tested isolates found to include a resistance gene for at least one antibiotic. In conclusion, more efficient interventions must be followed to control the redundant use of antibiotics in veterinary practice. Furthermore, appropriate control measures are needed to be implemented to reduce contamination and the spread of multiresistant *S. aureus* strains.

Keywords: *Antibiotic resistance, Animal origin foods, Multidrug resistance, Resistance genes, Staphylococcus aureus*

Hayvansal Gıdalardan İzole Edilen *Staphylococcus aureus*'ların Antibiyotik Dirençlilik Gen Profilleri

Öz

Bu çalışmada hayvansal gıdalardan izole edilen *Staphylococcus aureus* izolatlarının antibiyotik dirençlilik ve ilgili gen profillerinin araştırılması amaçlanmıştır. Besin Hijyeni ve Teknolojisi laboravuarımızda yer alan kültür koleksiyonunda, 2009 ve 2012 yılları arasında toplanan 95 *S. aureus* izolatları, fenotipik testler ve PCR ile doğrulanmıştır. İzolatların antibiyotiklere duyarlılıkları disk difüzyon testi ile, minimal inhibitor konsantrasyonları ise E test ile incelenmiştir. Ayrıca çalışmada PCR, *bla*Z, *erm*A, *erm*C, *tet*K, *tet*M, *mec*A, *Van*A, *Van*B, *Vat*A, *Vat*B ve *aacA-aphD* genlerinin varlığını tespit etmek amacıyla kullanılmıştır. Testler sonunda penisilin, tetrasiklin, vankomisin, eritromisin, sefoksitin, gentamisin ve quinupristin-dalfopristin antibiyotiklerine karşı direnç oranları sırasıyla %81.1, %28.4, %18.9, %17.9, %9.4, %9.4 ve %3.2 olarak bulunmuştur. E test sonuçları ile disk difüzyon bulguları birbiri ile uyumlu bulunmuştur. *S. aureus* izolatlarının çoklu ilaç direnci %29.5 bulunmuştur. Vankomisin dışında izolatların antibiyotiklere dirençlilikleri hususunda PCR ile konvansiyonel metotlar arasında uyum tespit edilmiştir. Ayrıca test edilen tüm izolatların en az bir antibiyotik için gen bulundurduğu gözlenmiştir. Sonuç olarak, veteriner pratikte gereksiz antibiyotik kullanımını kontrol etmek için daha etkin uygulamalar izlenmelidir ve çoklu ilaç direncine sahip *S. aureus* izolatlarının yayılımını ve bulaşını azaltmak için uygun kontrol önlemlerinin uygulanması gerekmektedir.

Anahtar sözcükler: *Antibiyotik direnci, Çoklu ilaç direnci, Direnç genleri, Hayvansal gıdalar, Staphylococcus aureus*

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INTRODUCTION

Staphylococcus aureus is a pathogen which is usually incriminated for a various kind of diseases ranging from skin infections to serious diseases, such as cellulitis, endocarditis and bacteremia [1-3]. Being commonly found on the skin and mucosae of food producing animal reservoirs, *S. aureus* is one of the most important worldwide food poisoning agents [1].

S. aureus developed resistance shortly after the introduction of penicillin in 1940s, followed by methicillin resistance in 1961. Hitherto, numbers of publication have been reported concerning the resistance of *S. aureus* isolates to diverse spectrum of antibiotics [3,4]. *S. aureus* is causing a concern due to its ability to become resistant to antibiotics via acquired by horizontal transfer of genes and chromosomal mutation [5]. Moreover, enzymatic drug modifications, changes in the target sites and membrane bound efflux pumps are additional mechanisms for the bacteria to combat against antimicrobial agents [6].

Multidrug-resistant (MDR) *S. aureus* isolates are of great public concern as it is possible that these resistant organisms can be transferred to humans via the food chain which in turn leads a limited choice for their control [7]. MDR *S. aureus* strains have frequently been reported from meat, dairy, fishery, poultry, eggs and salads [8-13].

The aim of this study was to characterize the recovered *S. aureus* isolates obtained from various foods of animal origin for their antimicrobial resistance by conventional and molecular methods.

MATERIAL and METHODS

Bacterial Strains

A total of 95 *S. aureus* strains, collected during a period between 2009 and 2012, were obtained from culture collection of the Food Hygiene and Technology Laboratory, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Turkey. These isolates were recovered from raw milk (n=12), sheep cheese (n=12), dairy dessert (n=11), chicken meat (n=12), pastrami (n=12), sausage (n=12), salami (n=12) and soudjouk (n=12). All isolates were confirmed as *S. aureus* by Gram staining, catalase activity, tube coagulase test and *nuc* gene amplification [14,15].

Antibiotic Susceptibility Test

In this study, antibiotic susceptibility testing was performed by disc diffusion method. The antibiotics investigated were (Oxoid, UK) gentamycin (CN, 10 µg), erythromycin (E, 15 µg), tetracycline (TE, 30 µg), penicillin G (P, 10 IU), cefoxitin (FOX, 30 µg), vancomycin (VA, 30 µg) and quinupristin-dalfopristin (QD, 15 µg). The minimal inhibition concentrations (MICs) of all above mentioned antibiotics for each isolates were determined by the E test (Oxoid, UK; Biomerieux, France and Liofil chem, Italy). The disc diffusion test results and MICs were interpreted using the criteria published by Clinical and Laboratory Standards Institute [16]. *Escherichia coli* ATCC 25922, *S. aureus* ATCC 29213 (for microdilution method) and *S. aureus* ATCC 25923 (for disc difusion method) were included as quality control strains in each run. The multidrug resistance was reported whether the single isolate is resistant to three or more unique antimicrobial classes.

DNA Extraction

Total genomic DNA was extracted from overnight-grown at 35°C *S. aureus* cultures in Brain Heart Infusion Broth (Acumedia, 7116A, USA) with the Genomic DNA Purification Kit (InstaGene™ Matrix, BIO-RAD, USA) as specified by the manufacturer.

Amplification of Nuc Gene

PCR assay conditions were used according to Cremonesi et al.[14] and carried out in a reaction mixture of 50 µL final volume containing, 5 µL of template DNA, 5 mL of 10x PCR buffer (Vivantis, Chino, CA), 2 U Taq polymerase (Vivantis), 2 mM dNTP mix (Vivantis), 1.5 mM $MgCl_2$ (Vivantis) and 30 mM of the primer pairs of each primer (NUC-F166 and NUC-R565). PCR conditions were: 5 min at 94°C for initial denaturation, 30 cycles of 1 min at 94°C, 1 min at 56°C for annealing and 1 min at 68°C for extension. The final extension was achieved 7 min at 72°C (Techne TC-512, Keison Products, Chelmsford, UK).

Detection of Selected Resistance Genes by PCR

The *bla*Z, *mec*A, *aac*A-*aph*D, *erm*A, *erm*C, *van*A, *van*B, *tet*K, *tet*M, *vat*A and *vat*B specific primer pairs were used for the amplifications of antibiotic resistance genes *(Table 1)*. PCR amplifications were performed with 50 µL PCR reaction mixture containing 5 µL of template DNA, 1x PCR Buffer (200 mM TrisHCl (pH 8.4), 500 mM KCl), 10 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 2.5 U Taq polymerase. The thermal cycling protocol for PCR was comprised initial denaturation at 94°C for 3 min, followed by 30 cycles of amplification with 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s with a final extension of 4 min at 72°C (Techne TC-512, UK) for *aac*A-*aph*D, *erm*A, *erm*C, *tet*K, *tet*M, *vat*A and *vat*B genes [17].

For *bla*Z gene, an initial step of 5 min at 94ºC was followed by 35 cycles of 1 min at 94ºC, 1 min at 54ºC and 1 min at 72ºC, and a final step at 72ºC for 10 min [18]. For the amplification of *mecA* gene, PCR reaction included an initial step at 94°C for 5 min followed by 35 cycles of 92ºC for 2 min, 55°C for 2 min, and 72ºC for 1 min with a final extension step at 72°C for 7 min [19]. For *vanA* and *vanB* genes an initial denaturation step at 94°C for 2 min; followed by 30 cycles of 94°C for 1, 54°C for 1 min

***Table 1.** The oligonucleotide sequence and predicted sizes used in the PCR*

Primer	Target Gen	Name	Sequence (5'-3')	Product Size (bp)	Anneling Temperature	Reference
Nuc-F166	*nuc*		AGT TCA GCA AAT GCA TCA CA	400	56°C	[14]
Nuc-R565			TAG CCA AGC CTT GAC GAA CT			
aacA-aphD-1	*aac*A-*aph*D	Gentamycin	TAA TCC AAG AGC AAT AAG GGC	227	55°C	[17]
aacA-aphD-2			GCC ACA CTA TCA TAA CCA CTA			
tetK-1	*tet*K	Tetracycline	GTA GCG ACA ATA GGT AAT AGT	360	55°C	[17]
tetK-2			GTA GTG ACA ATA AAC CTC CTA			
tetM-1	*tet*M		AGT GGA GCG ATT ACA GAA	158	55°C	
tetM-2			CAT ATG TCC TGG CGT GTC TA			
vatA-1	*vat*A	Quinupristin -Dalfopristin	TGG TCC CGG AAC AAC ATT TAT	268	55°C	[17]
vatA-2			TCC ACC GAC AAT AGA ATA GGG			
vatB-1	*vat*B		GCT GCG AAT TCA GTT GTT ACA	136	55°C	
vatB-2			CTG ACC AAT CCC ACC ATT TTA			
blaZ-1	*bla*Z	Penicillin G	TTA AAG TCT TAC CGA AAG CAG	377	54°C	[18]
blaZ-2			TAA GAG ATT TGC CTA TGC TT			
ermA-1	*erm*A	Erythromycin	AAG CGG TAA ACC CCT CTG A	190	55°C	[17]
ermA-2			TTC GCA AAT CCC TTC TCA AC			
ermC-1	*erm*C		AAT CGT CAA TTC CTG CAT GT	299	55°C	
ermC-2			TAA TCG TGG AAT ACG GGT TTG			
mecA-1	*mec*A	Cefoxitin	ACTGCTATCCACCCTCAAA	163	55°C	[19]
mecA-2			CTGGTGAAGTTGTAATCTGG			
vanA-1	*van*A	Vancomycin	GTAGGCTGCGATATTCAAAGC	231	54°C	[20]
vanA-2			CGATTCAATTGCGTAGTCCAA			
vanB-1	*van*B		GTAGGCTGCGATATTCAAAGC	330	54°C	
vanB-2			GCCGACAATCAAATCATCCTC			

and 72°C for 1 min and final extension at 72°C for10 min were done [20]. Amplification products were visualized by electrophoresis in 1.5% agarose gel (100 V for 40 min, EC250-90, Thermo, USA).

RESULTS

In the antibiotic susceptibility test, resistance to penicillin G (P), tetracycline (TE), vancomycin (VA) and erythromycin (E) was evident in 81.1%, 28.4%, 18.9% and 17.9% of the isolates used in this study, respectively. A small percentage of isolates demonstrated resistance to gentamycin (CN), cefoxitin (FOX) and quinupristin-dalfopristin (QD) with the rate of 9.4%, 9.4% and 3.2% respectively *(Table 2)*. The results of E test were compatible with that of disc diffusion method. In the E test, the ranges of MIC values were; gentamycin 0.06-256 μg/mL, erythromycin 0.03-128 μg/mL, cefoxitin 0.5-128 μg/mL, vancomycin 0.03-256 μg/mL, tetracycline 0.03-256 μg/mL, penicillin G 0.015-16 μg/mL and quinupristin-dalfopristin 0.008-32 μg/mL.

Multidrug Resistance

In this study, 28 (29.5%) of *S. aureus* isolates were resistant to three or more antibacterial classes. The majority of MDR *S. aureus* isolates (22.1%) were found resistant to three antimicrobials whereas resistances to four and six antimicrobials were observed from 5.2% and 2.1% of the isolates respectively *(Table 3)*.

Relationship Between Antibiotic Susceptibility Testing and PCRs

In this study, the antibiotic susceptibility test results and the presence of antibiotic resistance genes of 95 *S. aureus* isolates were compared *(Table 4)*. All nine gentamycin and cefoxitin resistant isolates (100%) were carried *aac*A-*aph*D and *mec*A genes, respectively. Nine (53%) and eight (47%) of 17 erythromycin resistant isolates were shown to have *erm*A and *erm*C genes, respectively. Seventy of 77 (91%) penicillin G resistant isolates carried *bla*Z gene. Although none of the isolates were found having *van*A gene, two of 18 vancomycin resistant isolates were carried *van*B gene. Twelve (44%) of 27 tetracycline resistant isolates had only *tet*K gene, 10 (37%) had only *tet*M gene while five (19%) had both *tet*K and *tet*M genes. All of three quinupristin-dalfopristin resistant isolates were found to harbour *vat*B gene *(Table 4)*.

***Table 2.** Antibiotic resistance profiles of S. aureus isolates*

Antibiotics	S		I		R		S (%)		I (%)		R (%)	
	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC
Gentamycin	≥15	≤4	13-14	8	≤12	≥16	79 (83.1)	79 (83.1)	7 (7.4)	7 (7.4)	9 (9.4)	9 (9.4)
Erythromycin	≥23	≤0.5	14-22	1-4	≤13	≥8	32 (33.7)	32 (33.7)	46 (48.4)	46 (48.4)	17 (17.9)	17 (17.9)
Penicillin G	≥29	≤0.12	-	-	≤28	≥0.25	18 (18.9)	18 (18.9)	-	-	77 (81.1)	77 (81.1)
Cefoxitin	≥22	≤4	-	-	≤21	≥8	86 (90.5)	86 (90.5)	-	-	9 (9.4)	9 (9.4)
Vancomycin	≥15	≤2	-	4-8	-	≥16	73 (76.8)	73 (76.8)	4 (4.2)	4 (4.2)	18 (18.9)	18 (18.9)
Tetracycline	≥19	≤4	15-18	8	≤14	≥16	68 (71.6)	53 (55.8)	-	15 (15.8)	27 (28.4)	27 (28.4)
Quinupristin-Dalfopristin	≥19	≤1	16-18	2	≤15	≥4	82 (86.3)	82 (86.3)	10 (10.5)	10 (10.5)	3 (3.2)	3 (3.2)

S: Susceptile; I: Intermediate; R: Resistance, DD: Disc Diffusion Test

***Table 3.** Multidrug resistances of S. aureus isolates*

Number of Resistances	Resistance Patterns	Number of Resistant Isolates
3	CN, P, VA	2
3	CN, E, VA	1
3	E, P, TE	3
3	E, P, VA	2
3	P, VA, TE	8
3	CN, E, P	1
3	P, VA, FOX	4
4	CN, E, P, TE	2
4	CN, P, VA, TE	1
4	CN, P, VA, FOX	1
4	P, VA, FOX, QD	1
6	CN, E, P, FOX, VA, QD	1
6	E, P, VA, FOX, TE, QD	1

CN: Gentamicin; E: Erythromicin, P: Penicillin; OX: Oxacillin; VA: Vancomycin; TE: Tetracycline, FOX: Cefoxitin; QD: Quinupristin-Dalfopristin

***Table 4.** Relationship between antibiotic susceptibility testing and PCRs*

Antibiotics	Target Gen	Resistance (%)	Gene Frequency (%)
Gentamycin	*aac*A- *aph*D	9 (9.4)	9/9 (100)
Tetracycline	*tet*K	27 (28.4)	12/27 (44)
	*tet*M		10/27 (37)
	*tet*K + *tet*M		5/27 (19)
Quinupristin-Dalfopristin	*vat*A	3 (3.2)	-
	*vat*B		3/3 (100)
Penicillin G	*bla*Z	77 (81.1)	70/77 (91)
Erythromicin	*erm*A	17 (17.9)	9/17 (53)
	*erm*C		8/17 (47)
Cefoxitin	*mec*A	9(9.4)	9/9 (100)
Vancomycin	*van*A	18 (18.9)	-
	*van*B		2/18 (11)

DISCUSSION

S. aureus is increasingly developing resistance to formerly effective antimicrobial agents [5,21]. Food is an important vehicle for the transfer of resistant *S. aureus* strains from animals to humans and antimicrobial resistant strains are emerging as a global problem. MDR *S. aureus* isolated from different foods exhibited a various distribution throughout the world [22,23].

In this study, remarkable levels (3.2-81.1%) of resistance to antibiotics which were all broad-spectrum, P, VA, TE, FOX, E, CN and QD *(Table 2)* were found. These results are not surprising for the antibiotics mentioned above as they are commonly used in both veterinary and human medicine.

In the present study, the resistance of gentamycin was found to be 9.4%. On the contrary, Groves et al.[24] and Gomes et al.[25] found higher results at rates of 83.4% and 26.8% from human isolates than that of ours. All gentamycin-resistant strains had the *aac*A-*aph*D gene similar to Strommenger et al.[17] (in Germany), Adwan et al.[26] (in Palestine) and Groves et al.[24] (in Australia) from clinical human isolates. In addition, Gomes et al.[25] (in Brazil) and Oksuz et al.[27] (in Turkey) reported that *aac*A-*aph*D gene was present in 43% and 95% of gentamycin-resistant isolates from clinical samples, respectively.

Erythromycin resistance was determined at rate of 17.9%. Moreover, we found nine (53%) *erm*A and eight (47%) *erm*C positive strains out of 17 erythromycin resistant isolates. In contrast, the frequency of erythromycin resistance (40%) was reported relatively high and related genes (*erm*A; 22.8% and *erm*C; 17.1%) was reported relatively low by Zmantar et al.[28] in *S. aureus* strains isolated from auricular infections in Tunisia. Moreover, Gao et al.[29] have also reported that erythromycin-resistant was evaluated as 44% and all erythromycin-resistant isolates to carry *erm*A and *erm*C genes from milk samples. In addition, Adwan et al.[26] stated that the presence of *erm*A and *erm*C genes among MRSA isolates were 30.9% and 74.5% from different clinical samples in Palestine. These inagreements

might be due to the source of isolates and mutation in the genes located in coding or promotor region of the PCR-detected genes or genes in small plasmids, seldomly lost. Moreover, the *erm*C gene encoding for ERM resistance is located on a small plasmid was reported by Fluit et al.[30]. Some reports indicated *erm*A to be more dominant factor in *S. aureus* infections [31,32].

In our study, the rate of penicillin G resistance was detected at 81.1%, the *bla*Z gene was present in 70 (91%) of 77 in resistant strains. However, Goa et al.[29] have reported penicillin resistance was 29%, the *bla*Z gene was present in 81% of them. Moreover, Yang et al.[33] reported that *bla*Z gene was detected in 94.6% of 37 penicillin resistant *S. aureus* strains isolated from bovine mastitis.

Regarding the methicillin-resistance and related gene (*mec*A), was detected in 9.4% and 100% of *S. aureus* isolates in this study, respectively. MRSA strains were detected by Pehlivanoglu and Yardimci [34], by Türütoğlu et al.[35] and by Sareyyupoglu et al.[36] in Turkey, by Kumar et al.[37] in India, by Moon et al.[38] in Korea, by Fessler et al.[39] in German. The rates of having *mec*A gene in milk samples have been reported as 77%,61.9%, 57%,16.7 % and 3.1% by Kumar et al.[37] in India, by Moon et al.[38] in Korea, by Pehlivanoglu and Yardimci [34], by Türütoğlu et al.[35] and by Sareyyupoglu et al.[36] in Turkey. Fessler et al.[39] have also reported that presence of *mec*A gene that has been observed is 37.2% in food and food products of poultry origin in German. The high rate of *mec*A gene obtained in this study, might be due to the horizontal transmission of this gene between the strains found together in food processing environment [40].

In our study, about 18.9% of the isolates were found to be resistant to vancomycin and 11% of the vancomycin resistant isolates harbored *van*B gene. Our results demonstrated that the presence of vancomycin resistance gene *(vanB)* in *S. aureus* strains isolated from food in Kayseri is of utmost importance. Therefore, further studies are necessary to keep the emergence and spread of these isolates. Similarly, Abulreesh [41] detected in 14% of 51 *S. aureus* isolates were resistant to vancomycin. Moreover, Pehlivanoglu and Yardimci [34], Turkyılmaz et al.[42] and McMillan et al.[43] reported that in milk samples, all strains were susceptible to vancomycin by disk diffusion test. Contrary to our study, Simeoni et al.[44] (from swine meat commodities in Italy), Pehlivanoglu and Yardimci [34] (from milk samples in Turkey), McMillan et al.[43] (from raw milk sources in Australia), and Abulreesh [41] (from potable water samples in Saudi Arabia) reported that none of the *S. aureus* isolates were found to be positive for *van*A/*van*B gene by PCR. The reasons for the different results might be explained with changes in biosynthesis of cell wall of the resistant strains [34,45,46].

The rate of quinupristin-dalfopristin resistant isolates were 3.2% and all resistant isolates had *vat*B gene in this study. Contrary, Adwan et al.[26] noted the prevalence of *vat*A was 1.8% and *vat*B gene was not found among MRSA isolates respectively. Fessler et al.[39] reported that from 86 samples originated from food and food products of poultry origin, four MRSA isolates were found to be resistant to quinupristin-dalfopristin, however 11 isolates were detected as intermediate. However, they detected that all isolates tested were negative for *vat*A or *vat*B gene.

In our study, no resistance genes were detected in some resistant isolates, which agreed with the findings of Gao et al.[47]. The phenotypic resistance may be caused by other resistance mechanisms, including biofilm formation, rather than gene acquisition [48]. The resistance mechanisms to antibiotics are so complicated that the presence or absence of a particular resistance gene cannot be regarded as a certain evidence for the isolate to be resistant or sensitive to the related antimicrobial agents [49].

In this study, a positive relation was observed between phenotypic and PCR results for the determination of antimicrobial resistance which is in agreement with Gao et al.[29] and Saadat et al.[50]. However, Zmantar et al.[28] and Salih et al.[51] reported no correlation between phenotypic and PCR methods.

According to MDR, 28 (29.5%) of *S. aureus* isolates were found resistant to three or more antimicrobial agents *(Table 3)*. Attention should be given to the fact that all of the isolates demonstrated resistance to more than one antibiotic. MDR has also been reported by Elbargisy et al.[52], Fan et al.[11], and Waters [53], at the rate of 17.1%, 66.3% and 52%, respectively. These findings might be due to the abundant use of antimicrobials for farm animals especially in countries where antibiotic use is not well regulated [11]. Large presence of MDR *S. aureus* in foods causes high risk of infections and a possible transmission of resistances to other pathogens which could lead to failure of antibiotic treatments.

In conclusion, spreading of MDR *S. aureus* via foodstuffs is a potential hazard for public health and might result in difficulties to treat MDR-related diseases. These results suggest that the incidence of MDR *S. aureus* are steadily increasing and attention needs to be paid to decrease or eliminate the contamination of MDR *S. aureus*. Specified education programs should be supported to define the prudent antibiotic use besides clinical guidelines should be developed and put into practice.

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