ORIGINAL ARTICLE

Molecular analysis of immune evasion cluster (IEC) genes and intercellular adhesion gene cluster (ICA) among methicillin-resistant and methicillin-sensitive isolates of *Staphylococcus aureus*

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Keywords

Staphylicoccus aureus • MRSA • MSSA • Biofilm • IEC types • Personal hygiene

Summary

Introduction. *Resistance to antibiotics and presence of virulence factors play an important role in increased mortality associated with infection due to Staphylococcus aureus. In this study, we determine antibiotic resistance pattern, presence of the icaADBC locus as well as biofilm formation and distribution and diversity the immune evasion cluster (IEC) genes in clinical isolate of S. aureus from Kerman, Iran.*

Materials and methods. *During 15 months, 100 clinical isolates S. aureus recovered from different patients were admitted to Kerman University affiliated hospitals. Resistance to different antibiotic agents was determined by disk diffusion method. Phenotypic method was used to the determination of biofilm formation ability and methicillin-resistance S. aureus (MRSA). Polymerase chain reaction technique (PCR) was used to the detection of nuc, mecA, icaA, icaD, icaB, icaC, scn, sea, sak, sep and chp genes..*

Results. *Forty-four isolates were considered as MRSA and all of isolates were sensitive to vancomycin and linezolid. Our results showed, 77.2% (34/44) of MRSA and 8.9 % (5/56) of MSSA isolates were multidrug resistant. The predominant IEC variant was type B and our results displayed that 77.7% of the MRSA isolates harbor loci icaD and mecA. There was no significant difference in production biofilm between MSSA and MRSA isolates ($P \geq 0.05$). There was significant difference in presence IEC types between MSSA and MRSA isolates ($P = 0.000$).*

Conclusions. *The presence of icaADBC locus may not be a determining factor for biofilm formation in Staphtlocooci and other mechanisms might be involved in this process.The high prevalence IEC types in MSSA isolates can indicate that the presence of these genes can be an advantage for pathogenesis of these isolates in different infections.*

Introduction

Staphylococcus aureus is both a commensal and a versatile human pathogen causing a broad spectrum of disease, from mild skin and soft tissue infections to life-threatening sepsis, pneumonia, endocarditis and deep-seated abscesses [1, 2]. The emergence of methicillin-resistant strains as well as having multiple virulence factors are the main factors in increased mortality in hospital–acquired (HA) and community-acquired (CA) infections caused by *S. aureus* [3]. Recent studies have displayed an increase in the worldwide prevalence of MRSA. In a regional perspective, a higher prevalence of MRSA in Iran compared to neighboring countries in the Middle East, except Iraq has been reported [4]. One study which reported HA-MRSA rates for eight Asian countries showed higher percentage of MRSA in those countries compared to Iran. Mean prevalence of MRSA in Iran is moderately higher than Australia and lower than the United States [5]. However, recent reports have revealed that MRSA rates are decreasing in United States [6]. This organism produces a number of virulence factors that provide the ability to colonize for it, adhere to surfaces as biofilm, invade or evade the immune system, develop resistance to multiple antibiotics and cause toxicity to the host [7, 8]. The ability of *S .aureus* to produce an extracellular slime and constitutive a biofilm enables this organism to withstand the host immune response and to make clinical treatment extremely difficult because of biofilm creation protects bacteria from antimicrobial agent [9]. The intracellular adhesion (*ica*) cluster, *icaADB* and *C*, encodes enzymes mediating cell-cell the adhesion and synthesis of the polysaccharide intercellular adhesion (PIA) which is essential for biofilm establishment [10, 11]. Another attribute of *S. aureus* which enables the pathogen's escape from protective immune responses express a number of immune-modulating proteins [12]. One of the immune-modulating proteins is staphylococcal complement inhibitor (SCIN). SCIN is a complement inhibitor, blocking the

ability of human neutrophils to opsonophagocytose of *S. aureus* and neutrophil chemotaxis [13, 14]. The gene encoding SCIN (*scn*) was found to be portion of a so-called immune evasion cluster (IEC). All IEC variants harbor *scn* and a different combination of *sak*, *chp* and *sea* (or *sep*). So far seven different variants of IEC carried by several different βC-Φs[14, 15]. These encode the human-specific immune modulators including staphylococcal enterotoxin A (SEA), staphylokinase (SAK) and chemotaxis inhibitory protein of *S. aureus* (CHIPS). SEA is involved in the down-regulation of chemokine receptors of monocytes [16]. SAK is a bacterial plasminogen activator and blocker, the bactericidal effect of antimicrobial peptides, the α-defensins [17]. CHIPS can bind to the formylated peptide receptor and neutrophils so can block neutrophil chemotaxis [18, 19].

To our knowledge, there is no information about prevalence of IEC-carrying βC-Φs in human *S. aureus* isolates in Iran. Since different studies have shown the decisive role of the *ica* genes as virulence factors in staphylococcal infections [20, 21] and IECs are bacteriophage encoded, the aim of this study was to determine antibiotic resistance pattern, the biofilm formation ability, the presence of the *icaADBC* locus and investigate the distribution and diversity the immune evasion cluster (IEC) genes among clinical isolate of *S. aureus* from Kerman, Iran.

Materials and methods

Bacterial isolates

A total of 100 non repetitive clinical isolates of *S. aureus* recovered from different patients, were admitted to Kerman University affiliated hospitals during February 2015 to May 2016. Clinical samples such as urine, blood, discharged abscess, wound, cerebrospinal fluid (CSF), broncoalveolar (BAL), synovial fluid and pus were included in this study. Bacterial isolates were considered as *S. aureus* by conventional biochemical standard methods including Gram-staining, catalase, slide and tube coagulase, DNase and maninitol fermentation on *mannitol salt agar* medium. All the applied culture media were purchased from Merck, Germany. The final identification of *S. aureus* isolates was done by amplification of *nuc* gene in species level in PCR method [22]. We defined 'community acquired (CA)' and 'healthcare associated infections or hospitalized patients (HA)' according to the current CDC criteria [23].

Susceptibility of isolates to antibiotics

The disk diffusion method on Müller-Hinton agar medium (MHA) was used to determine susceptibility of isolates to ciprofloxacin (5μg), trimethoprim/sulfamethoxazole (1.25/23.75μg), gentamicin (10μg), amikacin (30μg), erythromycin (15μg), clindamycin (2μg), tetracycline (30μg) and linezolid (30μg) (Mast disks,UK) according to guidelines of Clinical Laboratory Standards Institute (CLSI) [24]. The Brain Heart Infusion (*BHI*) agar medium with 6 μg/ml vancomycin used for screening of vancomycin resistant *S. aureus* isolates (VRSA). *S. aureus* ATCC 25923 was used as standard strain in susceptibility tests to antibiotics. Multidrug resistance (MDR) was defined as resistance of isolate to three or more unique antimicrobial drug classes in addition to beta-lactams [25].

Screening of methicillin-resistant *S. aureus* isolates (MRSA)

The MRSA isolates were detected by susceptibility of isolates to cefoxitin (FOX: 30 μg) on MHA according to recommendations of the CLSI [24] and then confirmed for the presence of *mecA* gene by PCR technique as described previously [26]. Patients who have acquired CA-MRSA infections did not have typical MRSA risk factors such as recent history of hospitalization, kidney dialysis, residence in a long-term health care facility or intravenous drug use.

Biofilm formation

S. aureus isolates were cultured on Trypticase Soy Agar (TSA) at 37ºC for 24h. Few grown colonies suspended in sterile physiological saline with turbidity equal to 0.5McFarland. The 96 well polystyrene microtiter platessterile (Cell and Tissue Culture plates, flat well bottom, Guangzhou Jet Bio-Filtration Products Co., Ltd. Guangdong, China) were filled with 180μl Trypticase Soy Broth (TSB) supplemented with 1% glucose and 20μl of bacterial suspension added to each well. After incubation for 24h at 37ºC, broth was carefully drawn off and the plates were gently washed three times with sterile phosphate-buffered saline (PBS). The plates were inverted and allowed to dry for 1 hour at room temperature. For biofilm quantification, 200μl of 2% saffranin dye solution in water was added to each well and the plates were allowed to stand for 40 min at room temperature. The wells were subsequently washed thrice with sterile PBS to wash off the excess saffranin. Saffranin bound to the biofilm was extracted with 200ml of 95% ethanol**,** and the absorbance of the extracted saffranin was measured at 490 nm in an ELISA reader (BioTek, USA). Each assay was performed in triplicate. As a negative control, TSB+1%glucose medium was used to determine background optical density (OD). The cut-off ODc for biofilm formation was determined as average OD of negative control +3×standard deviation (SD) of negative control. OD value was calculated for each microtiter plate separately. OD > 4×ODc was considered as high biofilm formation ability; 2×ODc < OD ≤ 4×ODc categorized into moderate biofilm formation ability. ODc < OD ≤ 2×ODc and OD ≤ ODc were taken as weak or none biofilm formation ability respectively [27].

DNA extraction

Deoxyribonucleic acid (DNA) extraction was performed by using appropriate DNA extraction kit (Gene All, Korea) following manufacturer's instruction. The quality of isolated DNA was measured by determination of absorbency at the wave length A260 nm and 280nm that showed a high quality of the product.

Tab. I. PCR primers and cycling parameters for genes presented in this study.

Gene target	Primer/sequence(5'-3')	PCR condition	PCR product (bp)	Reference
chp	F-GAAAAAGAAATTAGCAACAACAG R-CATAAGATGATTTAGACTCTCC	30 sec 95°C,50 sec 48°C,1min 72°C	410	[18]
sak	F-AAGGCGATGACGCGAGTTAT R-GCGCTTGGATCTAATTCAAC	30 sec 94 °C,30 sec 50 °C,1min 72 °C	223	[14]
sea	F-AGATCATTCGTGGTATAACG R-TTAACCGAAGGTTCTGTAGA	30 sec 94 °C,30 sec 50 °C,1min 72 °C	408	[14]
sep	F-AATCATAACCAACCGAATCA R-TCATAATGGAAGTGCTATAA	30 sec 94 °C,30 sec 50 °C,1min 72 °C	500	[14]
scn	F-AGCACAAGCTTGCCAACATCG R-TTAATATTTACTTTTTAGTGC	30 sec 94 °C,30 sec 49 °C,1min 72 °C	258	[14])
icaA	F- TCTCTTGCAGGAGCAATCAA R-TCAGGCACTAACATCCAGCA	1 min 95 °C,45 sec 60 °C,1min 72 °C	188	[28]
icaB	F- ATGGCTTAAAGCACACGACGC R- TATCGGCATCTGGTGTGACAG	1 min 95 °C,45 sec 61 °C,1min 72 °C	526	[29]
icaC	R- CTCTCTTAACATCATTCCGACGCC F- ATCATCGTGACACACTTACTAACG	1 min 95 °C,45 sec 63 °C,1min 72 °C	1013	[29]
icaD	F -GAACCGCTTGCCATGTGTTG R- GCTTGACCATGTTGCGTAACC	1 min 95 °C,45 sec 61 °C,1min 72 °C	483	[53]
mecA	F-TCC AGA TTA CAA CTT CAC CAG G R-CCA CTT CAT ATC TTG TAA CG	1 min 95 °C,45 sec 56 °C,1min 72 °C	162	[26]
nuc	F-GCGATTGATGGTGATACGGTT R-AGCCAAGCCTTGACGAACTAAAGC	1 min 95 °C,45 sec 60 °C,1min 72 °C	279	[30]

Detection of ICA and IEC cluster genes by PCR

Amplification was conducted in temperature gradient thermal cycler (Biometra-T300, Gottingen, Germany) in a volume of 25µl. Each 25µl PCR mixture consisted of 1µl of bacterial DNA, 0.5 µl (10pM) of each oligodeoxynucleotide primers, 12.5 µl of 2× Master Mix Red (Ampliqon, Denmark) and 11µl DNase and RNase free water. PCR was used for detection *nuc, mecA, icaA, icaD, icaB ,icaC ,scn, sea, sak, sep, chb* genes. All primers and programs can be found in Table I [14, 18, 26, 28-30]. After amplification, the PCR products were analyzed by electrophoresis on 1.5% agarose gel in 0.5×TBE buffer (5.4 g Tris base, 2.75 g boric acid, 2 ml 0.5 M EDTA, in 1 L). DNA ladder was a ready to use plasmid double digest sized range 100- 3000bp obtained from SMOBIO Technology (Hsinchu, Taiwan). Specificity of the primers was checked by Primer Quest software tool (http://www.ncbi.nlm.nih.gov/GenBank).

Statistical analysis

Statistical analysis was performed with SPSS (v.22.0) statistics software. We used the Chi-Square test for the comparison of our data. A difference was considered statistically significant at a P-value of < 0.05.

Results

A total of 100 *S. aureus* isolates were obtained from 80 in patients (HA) and 20 outpatients (CA) with different clinical infections. The isolates corresponded to 61 males and 39 females. Most of the clinical isolates belonged to urine 30% (n = 30) followed by wound 28% (n = 28), blood 22% (n = 22), BAL 7% (n = 7), CSF 3% (n = 3), skin abscesses, synovial fluid, pus and other clinical samples 10 %(n = 10). Of the 100 *S. aureus* isolates, 44 (44%) were MRSA which were recovered from urine (n = 22, 50%), wound (n = 9, 20.4%), blood (n = 4, 9.1%), BAL (n = 4, 9.1%), CSF (n =1, 2.3%) and pus (n = 3, 7%) samples. Among the MRSA isolates, 68.2 %(n = 30) were classified as hospital-acquired MRSA (HA-MRSA) and 31.8% (n = 14) as community-acquired MRSA (CA-MRSA).

Antimicrobial susceptibility testing and PCR results

The resistance profiles of MRSA and MSSA isolates to antimicrobial agents tested were listed in Table II. All of isolates were sensitive to vancomycin and linezolid. It was found that 93.2% (n = 41) and 86.3 %(n = 38) of MRSA isolates were resistant to erythromycin and tetracycline respectively. In addition, the highest resistance MSSA isolates was to trimethoprim/sulfamethoxazole 30.4% (n = 17) and tetracycline 28.6 %(n = 16). The resistance rates of MRSA isolates to amikacin, ciprofloxacin, clindamycin, erythromycin, gentamicin and tetracycline were significantly higher than among MSSA isolates (Table II). Three CA-MRSA isolates had intermediate (borderline) resistance to clindamycin, while 19 MSSA isolates had intermediate (borderline) resistance as follows: 3(5.3%) to ciprofloxacin, 6(10.7%) to clindamycin and 10 (17.9%) to erythromycin. Overall, 77.2% (34/44) of MRSA isolates and 8.9%(5/56) of MSSA were multidrug resistant (MDR). HA–MRSA isolates were more resistant to multiple antibacterial classes than CA-MRSA isolates (73.5% vs. 26.5%). Fifty (50%) isolates contained an IEC-converting (βC-Φs),

Tab. II. Antimicrobial profiles of MRSA (HA-MRSA and CA-MRSA) and MSSA isolates from 100 patients of Kerman University affiliated hospitals.

Type of isolates	Rate of resistance to antimicrobial agents. n(%)							
	Isolates. n(%)	AK	GM	CD	E	CIP	T	SXT
CA-MRSA	14 (14%)	3 (6.8)	6 (13.6)	9 (13.6)	14 (31.8)	6 (13.6)	11 (25)	6 (13.6)
HA-MRSA	30 (30%)	21 (47.7)	25 (57)	20 (45.5)	27 (61.4)	25 (57)	27 (61.4)	15 (34.1)
MSSA	56 (56%)	1 (1.8)	2 (3.6)	2 (3.6)	11 (19.6)	8 (14.3)	16 (28.6)	17 (30.4)
Total	100 (100)	25 (25)	33 (33)	31 (31)	52 (52)	39 (39)	54 (54)	38 (38)

AK; Amikacin, GM; Gentamicin, CD; Clindamycin, E; Erythromycin, CIP; Ciprofloxacin, T; Tetracycline, SXT; trimethoprim/sulfamethoxazole,

as demonstrated by the presence of *scn*. The predominant IEC variant was type B (*sak*, *chp* and *scn*) present in 20 (40%) of 50 clinical isolates. Variant A (*sea*, *sak*, *chp* and *scn*), C (*chp* and scn), D (*sea*, *sak* and *scn*), E (*sak* and scn), F (*sep*, *sak*, *chp*, *scn*), and G (*sep*, *sak* and scn) were present in 2 (4%), 5 (10%), 3 (6%), 14 (28%), 3 (6%) and 0 (0%) of the fifty clinical isolates, respectively. Three isolates have both *scn* and *sea* which were non type able and negative *mecA* (Tab. III). Concerning the virulence factors in all isolates, *chp* was present in 36 (36%), *sak* was in 60 (60%), and the super antigens *sea* and *sep* were in 10 (10%) and 3 (3%) respectively. *scn* was present in 54% of these isolates.There was significant difference in presence of IEC types between MSSA and MRSA isolates ($P = 0.000$) (Tab. III).

Biofilm formation

The ability to produce biofilm in 9 (9%) isolates was strong, 26 (26%) isolates was moderate, 48 (48%) isolates was weak and 17 (17%) of them had no production biofilm. The prevalence of *icaA, icaB, icaC* and *icaD* in all of isolates was 2%, 1%, 2% and 84% respectively. Only in one MSSA isolate, all *ica* genes were positive and biofilm was strong. There was no significant difference in production biofilm between MSSA and MRSA isolates ($P \geq 0.05$). Although 69 of 84 (82.1%) producing biofilm isolates were positive for *icaD*, no significance difference between the presence of *icaD* gene and biofilm production was observed ($P \geq 0.05$).Thirty four MRSA was *icaD* gene positive.

Discussion

S. aureus is a powerful pathogen that is able to grow in nearly any part of the human body. This bacterium remains the most frequent cause of hospital and community-acquired infections with the high prevalence and rapid spread of drug-resistant *S. aureus* strains. *S. aureus* generates biofilm and an array of immune evasion factors that protect it from innate immune defense system [31]. According to the results of this study, 44% of isolates recognized as MRSA, were positive for *mecA* gene. A study conducted by Javan et al. [32] reported 42.6 % frequency of MRSA in Tehran. However, the frequency of MRSA isolates in present study is more than the results of some previous reports published from Iran and some other countries [33-36].The estimated prevalence in our study was lower than that found in the studies of Khosravi et al. [37], Heidari et al. [38], Sepehriseresht et al. [39], Saderi et al. [40] and Gudarsi et al. [25]. A systematic review displayed that prevalence of MRSA in Iran is high and varies between 20.4% and 90% in different parts of the country [41]. Discrepancy in MRSA prevalence may reflect differences in infection control policies, origin of the isolates and the characteristics of the participants and hospital wards [25]. Rapid and correct determination of the different *S. aureus* isolated from patients is a major help in understanding the epidemiology of this bacteria and its infection control.

The full susceptibility of MRSA and MSSA isolates recovered from clinical samples to vancomycin and linezolid observed in this study, is possibly as a consequence of limited usage of these antimicrobial agents and indicates that these antimicrobial agents are effective for the treatment of *S. aureus* infections in our population. Our data are in agreement with susceptibility rates in Iran and other countries [40-43].

Majority of the MRSA were resistant to tetracycline and erythromycin and these resistance patterns have been documented already by another study [43]. In view of the high resistance rates of MRSA to gentamicin, clindamycin, tetracycline, ciprofloxacin and erythromycin antibiotics which are probably due to misuse and overuse of these antibiotics, display that empirical treatment of MRSA infections at our hospitals with these may not be effective. Hence, these antibacterial agents should no longer be considered first-line drugs for the treatment of MRSA infections in our population.Our study revealed that 30.4% of MSSA and 47.7% of MRSA isolates were resistant to trimethoprim/sulfamethoxazole. In contrast of our data, several studies have been reported low rates of resistance to trimethoprim/sulfamethoxazole in *S. aureus* isolates [25, 45]. Our data is not in agreement with study by Wang*et al* [46] who reported trimethoprim-sulfamethoxazole susceptibility rates of 78.6% and 95.3% for MRSA and MSSA isolates respectively, recovered from patients in 12 cities across China.

Tab. III. Distribution of IEC types among MSSA and MRSA isolates.

Type of isolates	Isolates. n (%)	No. of IEC types						
		A	B	C	D	E	F	G
MSSA	39 (69.6)	2	19	5	2	11	-	-
MRSA	8 (18.2)	-	1	-	1	3	3	-
Total	47 (47%)	2	20	5	3	14	3	-

In this study, 77.2% of MRSA isolates were multidrug resistant, and this is of concern. Our results emphasize the need for persistent monitoring of antimicrobial resistance development in *S. aureus* isolates that are involved in hospital-acquired infections.
MSSA isolates exhibited intermediate resistance to ciprofloxacin, clindamycin and erythromycin, suggesting that more isolates can become resistant in the near future and the possible antimicrobial therapies for infections associated with such staphylococcal strains are confined.
Presence of staphylococci, especially those strains which generate an extracellular slime and constitute a biofilm, making clinical treatment extremely difficult [47]. Our results displayed that 77.7% of the MRSA isolates harbor locus *icaD* and *mecA* gene. Frebourg *et al* [48] have demonstrated that a large proportion of clinical isolates carrying the *ica* locus also harbor the *mecA* gene. Similar results have been reported by Martin-Lopez *et al* [47] and mirzaee *et al* [49]. We noted that 7 MRSA and 5 MSSA isolates were *ica* genes negative and biofilm producers and that 5 MRSA and 10 MSSA isolates were *icaA* positive and biofilm negative. In this regard our data may support some published data based on that biofilm formation may rely on environmentally regulated, *icaADBC*-independent mechanism(s) in MRSA [50, 51]. Eftekhar et al. [52, 53] showed that biofilm formation is independent of the *icaADBC* carriage in clinical and skin isolates of *S. epidermidis*. In contrast, namvar [54] reported that *S. aureus* isolates had no ability to form biofilm, unless they were positive for *icaD* gene. Relationship between the biofilm formation and the presence of these *ica* genes has been demonstrated in other studies [55, 56]. From clinical viewpoint, explanation of the main adhesive mechanisms in infections may help in developing preventive and therapeutic criterions, such as antiadhesive coatings or antiadhesin medicines [28].
In the present study, we demonstrated that 50% clinical *S. aureus* isolates contained an IEC-carrying bacteriophage. The predominant IEC variant was type B which has reported as the predominant variant in human infectious isolates [14]. Immune evasion cluster (IEC) is known to playan important role in human colonization [15]. To our knowledge, this study is the first report of prevalence Immune Evasion Cluster (IEC) genes in clinical isolates of *S. aureus* in Iran.
One study has shown that 90% of the human clinical *S. aureus* strains from a genetically diverse collection contain an IEC-carrying βC-Φs [14]. Some studies have demonstrated that the high incidence of IEC- carrying βC-Φs compared to other mobile elements carrying virulence factors such as *eta*, *lukS-PV*/*lukFPV* which are also carried by bacteriophages in human *S.aureus* strains, is a unique feature [57, 58]. One major reason for this observation is probably due to ability IEC to carrying by several different phages so they can cover a huge host range. IEC has spread successfully through the *S. aureus* population and will continue to do so. This enables *S. aureus* with a unique mechanism to adapt to, and counteract, the human host [14].On the one hand knowledge of the virulence strategies can help choose new ways to combat staphylococcal infections. On the other hand identification virulence genes provides potential targets in the treatment of *S. aureus* infection. For example the potent capacityof CHIPS to inhibit neutrophil chemotaxis, in vitro and invivo, makes this protein a promising candidate anti-inflammatory drug for those diseases in which C5a-induced damage by neutrophils plays an essential role [18].
In conclusion,this study reports that there was no correlation between antibiotic resistance and biofilm formation in under study clinical isolates of *S. aureus* and the biofilm formation ability of several MRSA and MSSA isolates in the absence of *icaABCD* genes suggests that further investigation is necessary to better understand *ica*-independent biofilm formation mechanisms. Different IEC types were detected among the isolates but these types were absent in many MRSA isolates.The high prevalenceIEC types in MSSA isolates can indicate that the presence of these genes can be an advantage for pathogenesis of these isolates in different infections.

Acknowledgements

The authors would like to thank the staff of Department of Microbiology and Virology of Kerman University of Medical Sciences, Iran.
This work was supported by research council of Kerman University of Medical Sciences. Kerman, Iran (Grant No: 94/677).
The authors declare that there are no conflicts of interest associated with this manuscript.

Authors' contributions

DKN conceived and designed the research; RA and DKN equally contributed to drafting the article and analyzed data. SFLK and YF contributed in collecting and processing samples. All authors read and approved the final article.

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■ Received on March 7, 2017. Accepted on November 16, 2017.

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