Journal of Advanced Veterinary Research Volume 7, Issue 2 (2017) 33-38

Journal of Advanced Veterinary Research

http://advetresearch.com/index.php/avr/index

Prevalence of Toxigenic and Methicillin Resistant Staphylococci in Poultry Chain Production

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ARTICLE INFO

Original Research

Received:
13 February 2017

Accepted:
17 March 2017

Keywords:

CNS
Enterotoxin
MecA gene
Poultry
S. aureus

ABSTRACT

Staphylococci are a worldwide cause of human and animal infection and are considered to be of the most common causes of infections in birds. Enterotoxins produced by some staphylococcal species were recognized as a causative agent of staphylococcal food poisoning (SFP). Only enterotoxins produced by *Staphylococcus aureus* were as yet well characterized. Much less is known about enterotoxigenic potential of coagulase-negative species of genus Staphylococcus (CNS). It has been reported that enterotoxigenic CNS strains have been associated with human and animal infections and food poisoning. Samples collected from chicken production cycle (un hatched eggs, baby chicks, broilers, chicken meat and table eggs) in Luxor, Egypt were tested to investigate the presence of Staphylococcus species and detection of their enterotoxines genes with more special attention for detection of methicillin resistance gene (*mecA*). Samples were tested for *S. aureus* and CNS on the basis of cultural and biochemical properties and confirmed by PCR amplification of *16S rRNA* and *clfa* gene. Results showed that the presence of Staphylococci were 50/150 (33.3%), 14% of the samples were *S. aureus* (21/150), while, 19.33% were CNS (29/150). *mecA* gene was detected in 66.7% and 51.7% among *S. aureus* and CNS respectively. Enterotoxins genes (seb, *sec* and *see*) were found in 5 (23.8%) of *S. aureus* with a percent of (9.5%) for *seb* and *sec* and (4.8%) for *see*, while *sec* and *see* were found in 6 (20.6%) of CNS. With a percent (10.3%) for each.

Introduction

Staphylococcus spp. are significant bacteria in the etiology of avian diseases and may contaminate foods as a result of processed carcasses (Pepe *et al*., 2006). Although enterotoxins producing *S. aureus* is the most common cause of food-borne human illness throughout the world (Do Carmo *et al*., 2004), the other species such as *S. hyicus*, *S. sciuri*, *S. xylosus* or *S. cohnii* are also important, particularly because of carriage the genes encoding antimicrobial resistance (Aarestrup *et al*., 2000). Staphylococci are one of the most predominant groups during the slaughtering and processing of poultry, and they have been recovered from air samples (Ellerbroek, 1997), neck skin of chicken carcasses (Olivier *et al*., 1996), and machinery surfaces (Huys *et al*., 2005). By this reason contaminated poultry products could be the source of possible transmission of different staphylococci species including resistant strains to humans, during food processing at home. Staphylococcal food poisoning is caused by ingestion of enterotoxins preformed in the food contaminated essentially through human manipulation or raw material obtained from animals. Although coagulase-positive *Staphylococcus aureus* is the main agent responsible for food intoxication, some researches emphasise that coagulase-negative staphylococci (CNS) are able to produce staphylococcal enterotoxins and may be a potential cause of food poisoning (Da cunha *et al*., 2006). About 80%-90% of CNS isolates associated with hospital infections are methicillin-resistant coagulase negative staphylococci (MRCNS). So the aim of these study was to detect the occurrence of *Staphylococcus spp*. in chicken production cycle (Table eggs, unhatched eggs, baby chicks, broilers and chickens meat), As well as detection of *mecA* gene and entrotoxins production genes.

Materials and methods

Sample Collection

A total of 150 samples were collected from chickens and chickens byproducts from different farms and markets in Luxor city

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ISSN: 2090-6277/2090-6269/

(30 samples were collected from each type (unhatched eggs, baby chicks, broilers, chicken meat and table eggs).

Isolation of Staphylococci was done according to Sneath et al. (1986)

The collected samples were inoculated in BPW (Difco), cultured onto Mannitol Salt agar (Difco) then incubated for 24-48 hours at 37°C .The resulted colonies were examined for identifying morphological characteristic appearance of Staphylococcus species.

Identification and characterization of coagulase positive and negative Staphylococcus Species

The isolates were identified according to MacFaddin (2000) by using conventional techniques such as: catalase test, oxidase test, growth at 10% NaCl, Mannitol fermentation, coagulase test as well as using PCR by detection of 16rRNA gene specific for genus staphylococcus and *clfa* gene specific for *S. aureus*. (Mason *et al*., 2001).

Serotyping of Staphylococcus isolates

Coagulase negative staphylococci were selected and serotyped using INTEGRAL SYSTEM STAFILOCOCCHI kit based on biochemical tests (NCCLS, 2004).

Detection of enterotoxins and mecA by Polymerase chain reaction (PCR)

Extraction

All coagulase positive staphylococci CPS and CNS isolates were extracted according to QIAamp DNA mini kit (instructions (Qiagen, Germany).

Preparation of Master Mix

According to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit, the primers used have specific sequence and amplify specific products as shown in Table 1.

Cycling conditions of the primers during cPCR

Temperature and time conditions of the primers during PCR are shown in Table 2.

Results

A total of 50 (33.3%) isolates were identified as Staphylo-

Table 1. Oligonucleotide primers sequences used to detect specific genes

Gene	Primer	Primer sequence (5'-3')	Length of amplified product	Reference
Sea	GSEAF-1	GGTTATCAATGTGCGGGTGG	102 bp	Mehrotra *et al.* (2000)
	GSEAR-2	CGGCACTTTTTTCTCTTCGG		
Seb	GSEBF-1	GTATGGTGGTGTAACTGAGC	164 bp	
	GSEBR-2	CCAAATAGTGACGAGTTAGG		
Sec	GSECF-1	AGATGAAGTAGTTGATGTGTATGG	451 bp	
	GSECR-2	CACACTTTTAGAATCAACCG		
Sed	GSEDF-1	CCAATAATAGGAGAAAATAAAAG	278 bp	
	GSEDR-2	ATTGGTATTTTTTTTCGTTC		
See	GSEEF-1	AGGTTTTTTCACAGGTCATCC	209 bp	
	GSEER-2	CTTTTTTTTCTTCGGTCAATC		
mecA	MecA-F	GTA GAA ATG ACT GAA CGT CCG ATA A	310 bp	McClure *et al.* (2006)
	MecA-R	CCA ATT CCA CAT TGT TTC GGT CTA A		
16S rRNA	16S rRNA.F	CCTATAAGACTGGGATAACTTCGGG	791 bp	Mason *et al.* (2001)
	16S rRNA.R	CTTTGAGTTTCAACCTTGCGGTCG		
clfA	ClfA.F	GCAAAATCCAGCACAACAGGAAACGA	638 bp	
	clfA.R	CTTGATCTCCAGCCATAATTGGTGG		

Table 2. Temperature and time conditions of the primers during PCR.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
mecA	94°C 5 min.	94°C 30 sec.	50°C 30 sec.	72°C 45 sec.	35	72°C 7 min.
Enterotoxins genes	94°C 5 min.	94°C 30 sec.	50°C 45 sec.	72°C 45 sec.	35	72°C 10 min.
16S rRNA and clfA	94°C 5 min.	94°C 30 sec.	55°C 45 sec.	72°C 45 sec.	35	72°C 10 min.

coccus species, 14% of the samples were coagulase positive *S. aureus* (21/150), while, 19.33% were CNS (29/150). Coagulase positive *S. aureus* revealed from 16.7% (5/30) of table eggs, 20% (6/30) of un hatched eggs, 13.3(4/30) of baby chicks and 10% (3/30) of broilers and chicken meat, while CNS revealed from from10% (3/30) of table eggs, 26.7% (8/30) of unhatched eggs, 13.3% (4/30) of baby chicks, 33.3% (10/30) of broilers and 13.3% (4/30) of chicken meat as shown in Table 3 and Fig. 1.

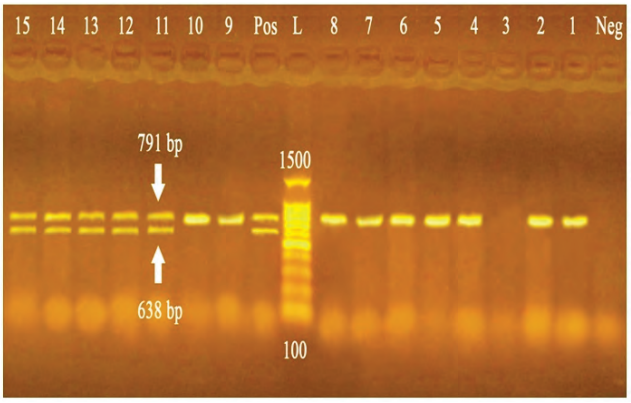

Fig. 1. PCR result for the *16S rRNA* gene (791bp) and *clfa* gene (638bp). Lane L: DNA ladder, Lane Neg; control –ve, Lane pos: control +ve (*S. aureus* strain), lane 3: non staph. Isolate, Lane 1,2 ,4,5,6,7,8,9 and10: CNS isolates, Lane 11.12.13.14 and 15 *S. aureus*.

The INTEGRAL SYSTEM STAFILOCOCCHI kit was used for identification of CNS isolates. The results were as follow, out of 29 CNS isolates,10 isolates were *S. xylosus* (34.49%), 5 *S. warneri* (17.25%),3 isolates of each of *S. epidermidis*, *S. saprophyticus*, *S. simulans* and *S. hominis* (10.34%) and 2 isolate of S. capitis (6.9%).

mecA gene was detected as 66.7% and 51.7% among *S. aureus* and CNS respectively and it was found in CNS isolate as follow *S. xylosus*, *S. warneri*, *S. epidermidis* and *S. capitis* with the perecentages 50%, 60%, 33.3%, 100% respectively and *S. simulans* and *S. hominis* with 66.7%.

Enterotoxins were found in 5 (23.8%) of *S. aureus* as following: *seb* and *sec* (9.5%) and *see* (4.8%) and found in 6 (20.6%) of CNS as following: *sec* and *see* (10.3%). *sec* was detected in 2 isolates of *S. xylosus* and 1 isolate of S. simulans. *see* was detected in *S. xylosus*, *S. warneri*, *S. simulans* as shown in Table 4 and Figs 2, 3.

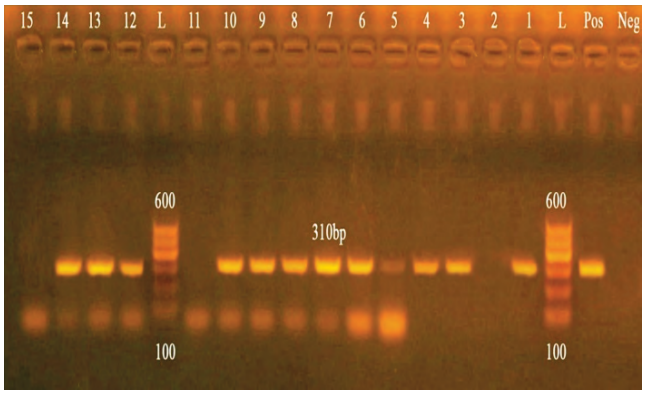

Fig. 2. PCR result of *mecA* gene among staphylococcus isolates. Lane L: ladder, lane pos: control positive, lane neg: control negative lane 1,3,4.5.6,7,8,9,10,12,13,14 (+ve mecA).lane 2,11,15 (-ve mecA)

Table 3. Occurrence of *S. aureus* and coagulase negative staphylococci from examined samples

Source of samples	No. of examined samples	Coagulase positive *S. aureus*		CNS		Total	
		No.	%	No.	%	No.	%
Table eggs	30	5	16.7	3	10	8	26.6
Unhatched eggs	30	6	20	8	26.7	14	46.6
Baby chicks	30	4	13.3	4	13.3	8	26.6
Broilers	30	3	10	10	33.3	13	43.3
Chickens meat	30	3	10	4	13.3	7	23.3
Total	150	21	14	29	19.33	50	33.3

Table 4. Occurrence of enterotoxin genes and *mecA* gene among the staphylococcus isolates.

	No. of examined samples	*seb*		*sec*		*see*		*mecA*	
		No.	%	No.	%	No.	%	No.	%
CPS *(S. aureus)*	21	2	9.5	2	9.5	1	4.8	14	66.7
S. xylosus	10	0	0	2	20	1	11.1	5	50
S. warneri	5	0	0	0	0	1	20	3	60
S. epidermidis	3	0	0	0	0	0	0	1	33.3
S. saprophyticus	3	0	0	0	0	0	0	0	0
S. simulans	3	0	0	1	33.3	1	33.3	2	66.7
S. hominis	3	0	0	0	0	0	0	2	66.7
S. capitis	2	0	0	0	0	0	0	2	100
Total	50	2	4	5	10	4	8	29	58

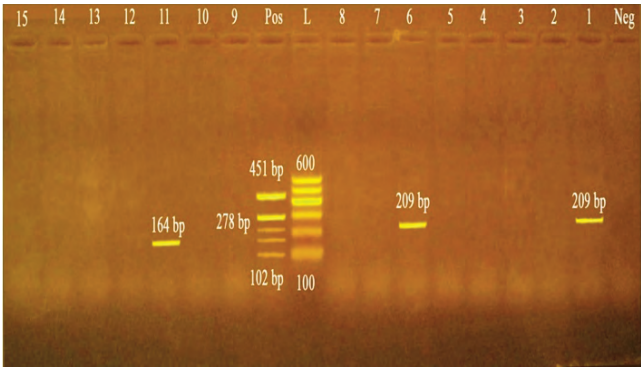

Fig. 3. PCR result for enterotoxin gene. *seb* (164 pb), *sec* (451 pb), *see* (209 pb) among staphylococcus species. Lane L: ladder, lane pos: control positive, lane neg: control negative lane 1and 6 (+ve *see*), lane 11(+ve *seb*).

Discussion

Chicken meat contributes substantially to the diet. Poultry meat products, especially chicken meat, are important, low-cost source of animal protein. This encourages the consumption of chicken meat by a large number of consumers. Recently, the consumption of chicken meat and chicken by-products was implicated in high number of outbreaks of staphylococcal food-poisoning in humans in different countries (Lee, 2006). Eggs can fully meet the requirements of all nutrients necessary for human development and life functions. At the same time, many nutrient substances present in egg create an excellent environment for the development of different microflora, including pathogenic microorganisms (Griffiths, 2005). *Staphylococcus spp*. are significant bacteria in the etiology of avian diseases and may contaminate foods as a result of processed carcasses (Pepe *et al*., 2006). In poultry, staphylococci, including *S. aureus* are known to cause various diseases from acute septicemia to chronic osteomyelitis. Staphylococcal osteomyelitis has been recognized as one of the major problems in broiler chickens (Skeeles, 1997).

A total 50 staphylococcal isolates were isolated from 150 samples of chicken production cycle in present study. 21(14%) isolates were identified as coagulase positive staphylococcus aureus and 29 (19.33%) isolates were identified as CNS by using staphylococci specific genus primer (16S rRNA) and *S. aureus* primer (clfa) (Mason *et al*., 2001). It is clear that percentage of CNS was high (58%) compared to CPS (42%) and this agreed with Goja *et al*. (2013); Yurdakul *et al*. (2013); Piyali and Pranab (2016), the latter found that the percentage of CNS (60%) was higher than that of CPS (40%). The high number of CNS isolated in this study could be justified by the fact that CNS are found abundantly in the normal teat skin flora and mucosa of humans and animals while some are free living in the environment (Addis *et al*., 2011)

In this study, CNS isolates were *Staphylococcus xylosus* (34.49%), *Staphylococcus warneri* (17.25%), *Staphylococcus epidermidis* (10.34%), *Staphylococcus saprophyticus* (10.34%), *Staphylococcus simulans* (10.34%) , *Staphylococcus hominis* (10.34%), *Staphylococcus capitis* (6.9%),that had been identified by INTEGRAL SYSTEM STAFILOCOCCHI KIT (NCCLS 2004) and biochemically according to MacFaddin (2000).This similar to Da cunha *et al*. (2006), who detected CNS strains from food samples, *S. epidermidis* (40%), *S. xylosus* (20%), *S. warneri* (20%), S. saccharolyticus (15%), and *S. hominis* (5%).

Results revealed that staphylococci were detected in 26.7% of table eggs; 16.7% for *Staphylococcus aureus* and 10% for CNS (6.66% *S. saprophyticus* and 3.34% *S. epidermidis* of CNS isolates and this agreed with Stepień *et al*. (2009) who found both coagulase-positive strains (*S. aureus* and *S. hyicus*) and coagulase-negative strains, particularly S. lentus, *S. warneri*, *S. epidermidis* and *S. xylosus* were isolated from whites, yolks and shells of eggs.

In the present study, staphylococci were isolated from 30 unhatched eggs (18 and 21 day) dead and live embryo in 14(46.67%).*S. aureus* were 6 (20%) and coagulase negative staphylococcus isolates were 8(26.7%). (37.5% *S. xylosus*, 12.5% *S. hominis*, 12.5% S.simulans, 12.5% *S. saprophyticus* and 25% *S. warneri*) but (Babaca, 2014) isolated staphylococcus species from dead-in-shell chicken in 21.6%.

S. aureus contamination is very important cause of arthritis in chicks and early chick mortalities (Abd El-Latif, 1995). Examination of baby chicks revealed that 8 (26.6%) staphylococcus species were isolated from 30 baby chicks (1-7days). 4 (13.3%) from each *S. aureus* and CNS. Coagulase negative staphylococcus isolates were 2 (50%) *S. xylosus*. 1 (25%) *S. saprophyticus* and 1(25%) *S. epidermidis*., and this result agreed with Abd El-Galil *et al*. (1984) and Azmy (1996) who isolated *S. aureus* from newly hatched at a prevalence of 14.7% and 15% respectively and disagreed with Al-khalaf *et al*. (2010), who isolated *S. aureus* from newly hatched chicks (5) out of 150 and Shareef *et al*. (2009) isolated *S. aureus* with a percentage of 29.1% from one day old chick samples.

Staphylococcal osteomyelitis has been recognized as one of the major problems in broiler chickens as reported by Skeeles (1997). In these study staphylococcus species were detected in 13 out of 30 (43.3%) from broiler, 3 (10%) were *S. aureus* and 10 (33.3%) strains were CNS (*S. xylosus* (5), S. simulans (2), S. capitis (2), *S. warneri* (1). and this agreed with Sobhy *et al*. (2014), who detected S. capitis, S. simulans, S. sciuri, S. haemolyticus, *S. xylosus* and *S. saprophyticus* from broilers chicken and agreed with Youssef and Hamed (2012), who isolated *S. aureus* (11.7%) from apparently health broilers in Ismailia governorate. On the other hand, they were inconsistent with the results of Rasheed (2011) that isolated S.aureus at the percentage of 50.98% from different broiler chickens farm, this may be due to the samples obtained from healthy and diseased bird.

The result of this study revealed that staphylococcus species found in 7 (23.3%) from 30 raw chicken samples (breast, neck and thigh), 3 (10%) strains were *S. aureus* and 4 (13.3%) were CNS (*S. hominis* (50%) and *S. warneri* (50%). the result agreed with Mohammad *et al*. (2014) that isolated *S. aureus* from15.7% raw chicken meat. also agreed with Sumru and Tugba (2011), who found CNS in 25.2% chicken. and disagreed with Yurdakul *et al*. (2013) who isolated 22 coagulase negative staphylococci from 50 chicken meat samples and this may be due to difference of the sample collection site.

It was observed that 66.7% (14/21) and 51.7% (15/29) of *S. aureus* and coagulase negative staphylococcus isolates (CNS) were positive for detection of *mecA* gene. The results were agreed with Febler *et al*. (2012), who isolated MRSA (Methicillin-resistant *Staphylococcus aureus*) from (50.0%) of staphylococcus isolates, Helen *et al*. (2011) detected MR-CNS in 48.3% of examined samples and Koksal *et al*. (2009), who observed that 67.5% of CNS isolates were Methicillin-resistant, while the result disagreed with Akbar and Anal (2013), who detected *mecA* gene in 18.18%, and EL-Shareek and Ali (2012) that found the gene in 29.6 %.

Staphylococcal enterotoxins (SE) constitute a family of biologically and structurally related toxins and the ingestion of these toxins results in gastrointestinal effects such as nausea, vomiting, diarrhea and abdominal pain. The SEs are the main cause of many outbreaks of food borne diseases (Lamaita *et al*., 2005)

Although enterotoxins are produced mainly by coagulase positive staphylococci, some coagulase-negative staphylococci (CNS), involved in a variety of human and animal infections (Kloos *et al*., 1995), CNS can contaminate foods because

humans are common carriers of these microorganisms and some may be related to specific human infections (Bergdoll, 1995).

The data illustrated that 5/21 *S. aureus* isolates (23.8%) and 6/29 CNS strains (20.7%) showed positive results for presence of enterotoxin genes. Three classical enterotoxin genes (*seb*, *sec* and *see*) were detected in the present work. This was agreed with Kitai *et al*. (2005) (21.7%); Naffa *et al*. (2006) (23%) ; Holmberg and Blake (1984), (26.5%) of *S. aureus* isolates produced SE and Çepoğlu *et al*. (2010), who found that 20% of 20 CPS was produced staphylococcal enterotoxin.

In this study *seb* enterotoxin was produced by 9.5% of *S. aureus* and was not produced by CNS. The result agreed with Polledo *et al*. (1985), who found that the distribution of enterotoxins *seb* were 8 (9.6%) of CPS strains and disagreed with Kitai *et al*. (2005), who detected *seb* in 64.1% of *S. aureus* and Rasoul *et al*. (2015) in 4.1%. Staphylococcal enterotoxin B (SEB) is the toxin most commonly associated with classic food poisoning. CDC (2014) reported that SEB has been studied as a potential biological warfare agent because it can easily be aerosolized; it is very stable; and can cause widespread systemic damage, multi-organ system failure, and even shock and death when inhaled at very high dosages. However, SEB is classified as an incapacitating agent because in most cases aerosol exposure does not result in death but in a temporary, though profoundly incapacitating, illness lasting as long as 2 weeks (Ulrich *et al*., 1997).

Studies have shown that *sec* is the most thermostable enterotoxins, followed by *seb* and sea (Notermans *et al*., 1988), while *sec* enterotoxin was found in this study by 9.5% and 10.3% of *S. aureus* and CNS respectively. This agreed with Polledo *et al*. (1985), who found *sec* in 8.4% from CPS strains and Kitai *et al*. (2005), who found *sec* in 10.3% of *S. aureus* and disagree with Enas *et al*. (2016) that detected *sec* gene in 23% of *S. aureus* isolates.

Staphylococcal food poisoning outbreak where *see* has been confirmed as the causative agent (Ostyn *et al*., 2010). In this study *see* enterotxin was detected in 4.8% and 10.3% from *S. aureus* and CNS respectively, this was agreed with Holmberg and Blake (1984), who detected *see* in 4.3% of S.aureus . However, Asadollahi *et al*. (2014) who reported a high level of *see* gene (31%). Other studies reported low level of *see* gene distribution (2.4%) (Polledo *et al*., 1985).

The SE genes *seb* and *sec* were found in similar percentages (Rall *et al*., 2010b), which agreed with results from this study, which also demonstrated the presence of *sec* and *see* genes in 30% of *S. xylosus*, which agreed with Da cunha *et al*. (2006), who detected *sec* gene in one *S. xylosus* isolate. *S. xylosus*, are used as a starter culture in fermented meat products (Montel *et al*., 2000)

In present study *S. warneri* carried *see* enterotoxin gene in 20%. S. simulence produced *sec* and *see* enterotoxun in 33.3%. However, *S. epidermidis*, *S. saprophyticus*, *S. hominis* and *S. capitis* not produced enterotoxins.

Valle *et al*. (1991) found a toxigenic capacity in 45 (16.5%) CNS isolates, including *S. epidermidis*, S. haemolyticus, *S. warneri*, and *S. xylosus*, Stępień *et al*. (2016) suggested a strong association between coagulase-negative *S. simulans* and endocarditis in broiler chicken.

Conclusion

From above mentioned data, it was observed that coagulase positive Staphylococci and coagulase negative staphylococci contain MecA gene and different types of enterotoxin genes. So, attention should be given to CNS because there is no more studies on it.

Acknowledgement

Many thanks to Dr. Waleed Younis, Assistant lecturer of Microbiology. Dept. of Microbiology. Faculty of Veterinary Medicine, South Valley University, Qena, Egypt. For his technical support.

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