

Enterotoxin genes, multidrug resistance, and molecular typing of *Staphylococcus* spp. isolated from organic bovine milk

Genes de enterotoxinas, multirresistência a antimicrobianos e caracterização molecular de espécies de Staphylococcus spp. isoladas de leite bovino orgânico

Amanda Keller SIQUEIRA¹; Tatiana SALERNO¹; Gustavo Henrique Batista LARA¹; Larissa Anuska Zeni CONDAS¹; Valéria Cataneli PEREIRA²; Danilo Flávio Moraes RIBOLI²; Fernando José Paganini LISTONI¹; Aristeu Vieira da SILVA³; Domingos da Silva LEITE⁴; Maria de Lourdes Ribeiro de Souza da CUNHA²; Márcio Garcia RIBEIRO¹

¹ Universidade Estadual Paulista “Júlio de Mesquita Filho”, Faculdade de Medicina Veterinária e Zootecnia, Departamento de Higiene Veterinária e Saúde Pública, Botucatu – SP, Brazil

² Universidade Estadual Paulista “Júlio de Mesquita Filho”, Instituto de Biociências, Departamento de Microbiologia e Imunologia, Botucatu – SP, Brazil

³ Universidade Estadual de Feira de Santana, Departamento de Ciências Biológicas, Feira de Santana – BA, Brazil

⁴ Universidade Estadual de Campinas, Instituto de Biologia, Departamento de Genética, Evolução e Bioagentes, Campinas – SP, Brazil

Abstract

The multidrug resistant and the emergence of methicillin-resistant staphylococci isolated from animals, food, and humans are public health concern. These microorganisms produce different toxins related to food poisoning in humans. This study aimed to characterize *Staphylococcus* spp. isolated from two organic milk farms in Brazil. A total of 259 milk samples were collected, from which 58 (22.4%) *Staphylococcus* spp. were isolated. The highest sensibility to ceftiofur and sulfamethoxazole/trimethoprim was observed in 96.6% of *Staphylococcus* spp., and whereas 89% were resistant to penicillin G. The *mecA* gene was detected in 13.8% of the isolates. SEA and SEC were the most common enterotoxins detected. PFGE revealed genetic heterogeneity from *S. intermedius* and *S. warneri* analyzed, while *S. aureus* presented similar profiles among isolates from the two studied herds. To the best of our knowledge, the current study describes for the first time presence of enterotoxins, *mecA* gene, and genetic diversity of staphylococci isolated from organic dairy farms in Brazil.

Keywords: Organic herd. Multidrug-resistance. *mecA*. PFGE. CNS.

Resumo

A emergência de estafilococos multirresistentes e resistentes à meticilina, isolados de animais, alimentos e humanos é uma preocupação em saúde pública. Esses micro-organismos produzem diferentes toxinas relacionadas à intoxicação alimentar em humanos. Este estudo caracterizou *Staphylococcus* spp. isolados em duas fazendas orgânicas no Brasil. Foram coletadas 259 amostras de leite em duas propriedades leiteiras orgânicas, nas quais 58 (22,4%) estirpes de *Staphylococcus* spp. foram isoladas. A maior sensibilidade dos isolados foi observada para ceftiofur e sulfametoxazol/trimetoprim em 96,6%. Em contraste, acima de 89% de resistência dos estafilococos foi encontrada para penicilina G. O gene *mecA* foi identificado em 13,8% dos isolados. SEA e SEC foram as enterotoxinas mais comumente detectadas. PFGE revelou heterogeneidade genética entre *S. intermedius* e *S. warneri*, enquanto *S. aureus* demonstraram perfis semelhantes entre isolados dos dois rebanhos estudados. Relata-se pela primeira vez no Brasil a detecção de enterotoxinas, o gene *mecA* e diversidade genética em estafilococos isolados de vacas em produção orgânica.

Palavras-chave: Rebanho orgânico. Multirresistência. *mecA*. PFGE. SCN.

Correspondence to:

Márcio Garcia Ribeiro
Universidade Estadual Paulista “Júlio de Mesquita Filho”,
Faculdade de Medicina Veterinária e Zootecnia, Departamento
de Higiene Veterinária e Saúde Pública
CEP 18618-681, Botucatu, SP, Brazil
E-mail: mgribeiro@fmvz.unesp.br

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The worldwide demand for organic products, especially dairy products, has increased and the main reasons for that include the consumers' perception regarding the environment in this kind of production, the desire to avoid the use of industrial chemical compounds such as pesticides and antimicrobials, maintenance of animal welfare, and the belief that organic foods are safer and more nutritious (ŚREDNICKA-TOBER et al., 2016).

In Brazil, despite the exponential increase on dairy consumption, the sector has deficiencies in the production and the quality of raw milk, mainly caused by mastitis and hygiene deficiencies, losing competitiveness in the international market (RIBEIRO et al., 2009). Mastitis in cows is caused predominantly by bacteria, especially *Staphylococcus* spp. These animals can become reservoirs of resistant microorganisms and spread them by direct or indirect contact (HAMMERUM; HEUER, 2009). Furthermore, some species like *Staphylococcus* coagulase-negative were frequently found in the milking environment and teat apex (DE VISSCHER et al., 2015) and can also harbor resistance and/or toxin genes, becoming reservoirs of the infectious pathogens.

Antimicrobial resistance is an emerging problem regarding these bacteria isolated from animals and humans. The report of Methicillin-resistant *Staphylococcus* (MRS) in dairy products and food animals (LEE, 2003) highlight the impact that multidrug-resistant bacteria isolated from cows can generate and the need to develop studies focused on the sensitivity profile of *Staphylococcus* spp. obtained from animals. In addition, these bacteria were frequently related to food poisoning (CUNHA et al., 2004), as 30 to 50% of *S. aureus* strains were able to produce these toxins (BALABAN; RASOOLY, 2000).

Because of the paucity of information concerning organic milk in Brazil, the present study aimed at investigating antimicrobial susceptibility, multidrug resistance, the presence of *mecA* and enterotoxin genes (*sea*, *seb*, *sec* and *sed*), and the relation between *Staphylococcus* spp. isolated from bovine milk from two certified organic dairy farms, farm 1 (SC) and farm 2 (NS), in two cities in the state of São Paulo, Brazil, 300 Km apart from each other. From these farms, 259 individual milk samples were collected between August 2008 and August 2009, in a total of 13 samplings (I – XIII) per herd of Holstein and Jersey cows, divided in 15mL sterile plastic vials after aseptic preparation of the mammary quarters. Samples were cultivated on defibrinated bovine blood agar (5%) and MacConkey agar. Plates were incubated aerobically at 37°C for 72 hours, with evaluations every 24 hours.

The bacteria were identified based on conventional phenotypic characteristics and according to a modified method (CUNHA et al., 2004) to differentiate coagulase-negative staphylococci (CNS) species. *Staphylococcus* spp. were submitted to the disc diffusion method (CLSI, 2008,

2012) using antimicrobial agents commercially available and widely recommended in the therapy of bovine mastitis. Oxacillin M.I.C Evaluator Strips^a (256 – 0.015 µg/mL) were also used to identify methicillin-resistance and the results were interpreted according to Clinical and Laboratory Standards Institute (2012). *S. aureus* ATCC 33591 (MRSA) and *S. aureus* ATCC 25923 (susceptible strain) were used as quality controls.

PCR reactions to detect methicillin-resistance gene (*mecA*) were performed according Murakami et al. (1991). Enterotoxins genes and *tsst-1* gene were detected employing the parameters described by Johnson et al. (1991), with some modifications proposed by Cunha et al. (2006). The reference strains *S. aureus* ATCC 13565 for *sea*, *S. aureus* ATCC 14458 for *seb*, *S. aureus* ATCC 19095 for *sec*, *S. aureus* ATCC 23235 for *sed*, and *S. aureus* ATCC 51650 for *tsst-1* were used as positive controls.

A protocol was done by *SmaI* nuclease digestion followed by PFGE (MCDUGAL et al., 2003) in order to investigate the relation between *Staphylococcus* spp. isolated from organic milk.

From 259 milk samples collected from two organic dairy farms (samplings I to XIII), 58 *Staphylococcus* spp. were identified, 86 samples from farm 1 (SC) and 173 from farm 2 (NS). Forty-eight (82.8%) of these samples were classified as coagulase-positive staphylococci-CPS (*S. aureus*, *S. hyicus*, *S. intermedius* and *S. schleiferi coagulans*), and 10 (17.2%) as CNS. Three species of CNS (*S. capitis urealyticus*, *S. epidermidis*, and *S. warneri*) were identified using a phenotypic method, but considering that the accuracy of phenotypic tests to identify CNS have been discussed in the last decade, molecular methods were proposed as preferred methods to classify them at species level (DE VISSCHER et al., 2015).

On the one hand, Zadoks and Watts (2009) recommended generalizing as “coagulase-negative *Staphylococcus* species” especially when only phenotypic methods were applied. On the other hand, De Visscher et al. (2015) did not recommend studying CNS as a single group mainly because of different pathogenicity factors associated to each species and also highlighted the importance of epidemiological studies at the species level to better understand the impact of these bacteria on milk production. The characterization of *Staphylococcus* spp. based on coagulase test was reliable and did not

^a M.I.C.E™. Oxoid.

generate doubts in interpretation. However, although the method used for the definition of CNS at species level is not suitable today, at the time of development of this research we did not have other tools available for that purpose.

The highest *in vitro* sensibility of the isolates observed was to oxacillin – 88.2% of the CPS and 87.5% of the CNS. Penicillin G and ampicillin were the least effective antimicrobials, since 100% and 62.5% of the CPS and CNS isolates were resistant to penicillin G, respectively; and

64.7% of the CPS were resistant to ampicillin (Table 1). Multidrug resistance to three or more antimicrobials was observed in 15.5% of the samples. A total of 4/58 (6.9%) *Staphylococcus* spp. were classified as MRS by phenotypic methods, two coagulase-positive (*S. aureus* and *S. intermedius*) and two CNS (*S. epidermidis* and *S. warneri*). The *mecA* gene was detected in seven CPS, six *S. intermedius* and one *S. aureus*, and in one CNS (*S. epidermidis*). From these, only *S. warneri* resistant to cefoxitin attested negative to *mecA* gene.

Table 1 – *In vitro* antimicrobial profile of 48 coagulase-positive *Staphylococcus* spp. and 10 coagulase-negative *Staphylococcus* spp. isolated from bovine organic milk

Organism	CPS									CNS											
	<i>S. intermedius</i> (n = 27)			<i>S. aureus</i> (n = 17)			<i>S. schleiferi</i> <i>coagulans</i> (n = 3)			<i>S. hyicus</i> (n = 1)			<i>S. warneri</i> (n = 8)			<i>S. epidermidis</i> (n = 1)			<i>S. capitis</i> <i>urealyticus</i> (n = 1)		
Antimicrobial	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)
Ampicillin	11.2	18.4	70.4	23.5	11.8	64.7	66.7	0	33.3	100	0	0	100	0	0	100	0	0	0	0	100
Ceftiofur	100	0	0	88.2	11.8	0	66.7	33.3	0	100	0	0	100	0	0	100	0	0	100	0	0
Enrofloxacin	55.5	33.3	11.2	94.1	5.9	0	33.3	33.3	33.3	100	0	0	75.0	25.0	0	100	0	0	0	100	0
Gentamicin	92.6	7.4	0	94.1	0	5.9	66.7	0	33.3	100	0	0	100	0	0	0	0	100	100	0	0
Neomycin	77.9	18.4	3.7	82.4	17.6	0	66.7	0	33.3	100	0	0	100	0	0	0	0	100	100	0	0
Oxacillin	96.3	0	3.7	88.2	0	11.8	100	0	0	100	0	0	87.5	0	12.5	0	0	100	100	0	0
Penicillin G	0	0	100	0	0	100	0	0	100	100	0	0	37.5	0	62.5	0	0	100	0	0	100
Sodium Cefoperazone	40.8	55.5	3.7	35.4	47.0	17.6	33.3	66.7	0	100	0	0	87.5	12.5	0	0	100	0	0	100	0
SXT	96.3	0	3.7	100	0	0	66.7	0	33.3	100	0	0	87.5	0	12.5	100	0	0	100	0	0

* CPS: Coagulase-positive staphylococci; CNS: Coagulase-negative staphylococci. Susceptibility test category, S: sensitive; I: intermediate; R: resistant. SXT: Trimethoprim/Sulfamethoxazole

The high prevalence of bovine staphylococcal mastitis in Brazil, and the recent introduction of organic procedures in some Brazilian farms probably contribute to the maintenance of multi-resistant *Staphylococcus* in organic milk system (RIBEIRO et al., 2009). In this scenario, our results from resistance and multidrug-resistance corroborated Walther and Perreten's (2007), and showed

that staphylococci collected from cows under organic production may harbor resistant genes, especially in Brazil where the organic dairy production system has been recently established.

In this study it was observed that PCR might be used to detect the *mecA* gene, since it proved more sensitive – 13.8% of the staphylococci were positive – when compared

to the phenotypic methods, according to which 6.9% were positive. Agreeing with Murakami et al. (1991) PCR could be considered the gold standard method in the detection of that gene.

Nevertheless, until now there are few studies involving *mecA* gene detection of staphylococci isolates from organic milk, particularly in Brazil. In a Swiss report, the authors described methicillin-resistant *S. epidermidis* isolated from a cow under organic system (WALTHER; PERRETEN, 2007). Coincidentally, in the current study *mecA* gene associated to phenotypic resistance to oxacillin was identified in one *S. epidermidis*.

Table 2 shows the results regarding the presence of genes encoding toxins in *Staphylococcus* spp.. The *sea* gene was detected in 32.7% of the staphylococci, *seb* and *sed* in 3.4%, and *sec* in 39.6%. None of the strains presented *tsst-1* gene. Staphylococcal enterotoxins (SE) are responsible for food poisoning around the world (CUNHA et al., 2006) and the isolation of enterotoxigenic *Staphylococcus* spp. from dairy milk has been reported. Similar to this study, in Brazil, but under conventional production system, Guimarães et al. (2013) described enterotoxin genes associated with their isolates, 66% of the CNS and 35% of the CPS isolated from milk of dairy cows were positive to the enterotoxins genes *sea*, *seb*, and *sec*.

Table 2 – Enterotoxin genes in *Staphylococcus* spp. isolated from organic dairy milk

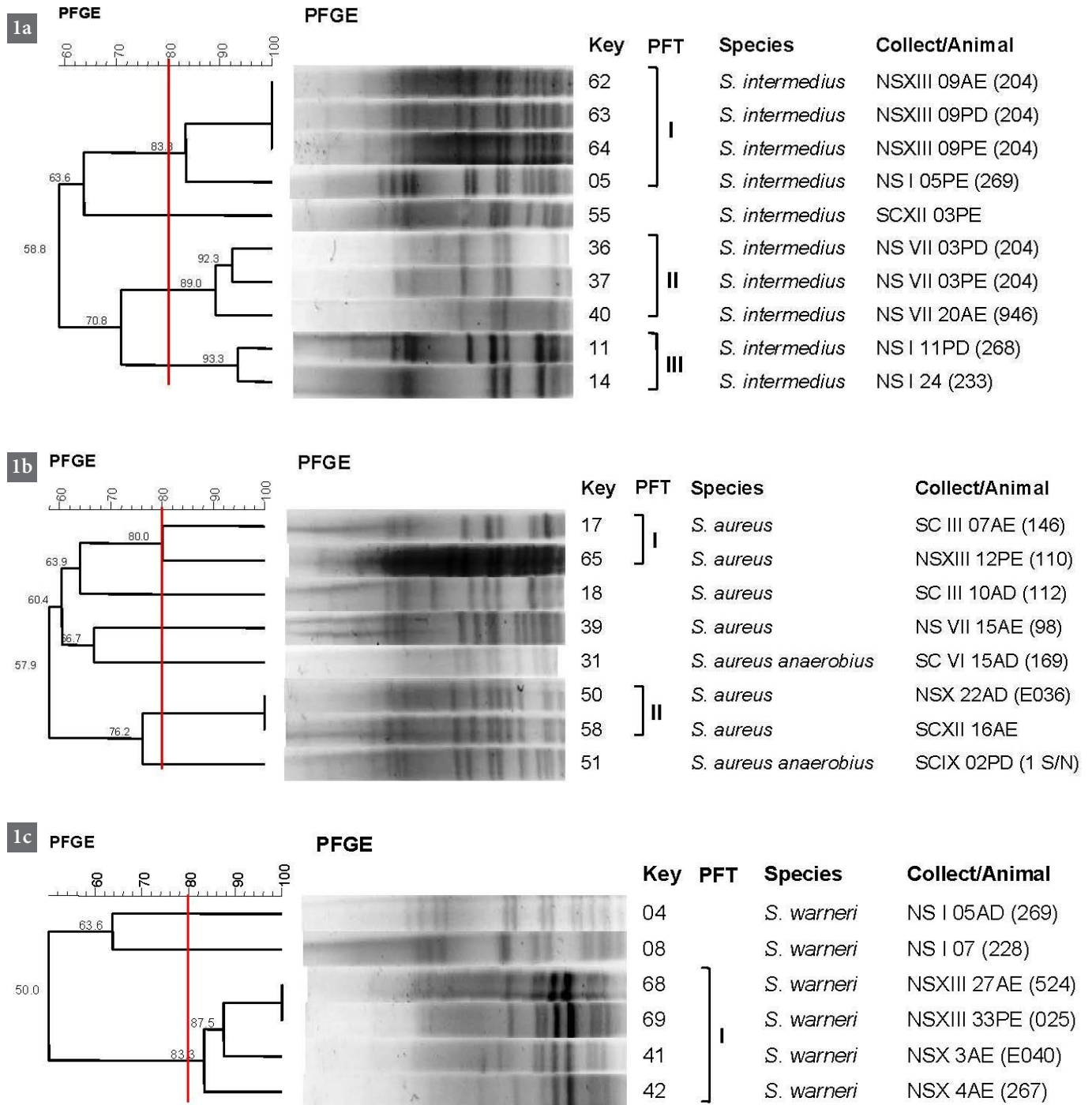
Genes	<i>*sea</i>	<i>seb</i>	<i>sec</i>	<i>sed</i>
	n strains/ total n of strains (%)	n strains/ total n of strains (%)	n strains/ total n of strains (%)	n strains/ total n of strains (%)
CPS				
<i>S. aureus</i>	5/17 (29.4)	1/17 (5.9)	4/17 (23.5)	1/17 (5.9)
<i>S. intermedius</i>	8/27 (29.6)	1/27 (3.7)	13/27 (48.1)	1/27 (3.7)
<i>S. schleiferi coagulans</i>	1/3 (33.3)	-	1/3 (33.3)	-
<i>S. hyicus</i>	1/1 (100.0)	-	-	-
CNS				
<i>S. warneri</i>	3/8 (37.5)	-	4/8 (50.0)	-
<i>S. epidermidis</i>	1/1 (100.0)	-	1/1 (100.0)	-
<i>S. capitis urealyticus</i>	-	-	-	-
Total	19/58 (32.7)	2/58 (3.4)	23/58 (39.6)	2/58 (3.4)

* *sea*: enterotoxin A gene; *seb*: enterotoxin B gene; *sec*: enterotoxin C gene; *sed*: enterotoxin D gene; n: number; CPS: Coagulase-positive staphylococci; CNS: Coagulase-negative staphylococci

Of the 24 isolates typed by PFGE, ten *S. intermedius*, eight *S. aureus*, and six *S. warneri* were analyzed per group. Three genetic profiles of *S. intermedius*, two of *S. aureus*, and one of *S. warneri* with a coefficient of similarity $\geq 80\%$ were identified. The PFGE analysis of ten *S. intermedius* revealed the persistence of one pulsotype along one year in the NS herd (PFTI). Other

two pulsotypes were observed in the same herd (NS), in different cows (animal numbers 204, 946, 268 and 233) (Figure 1a). Eight *S. aureus* presented two distinct PFGE patterns (PFTI and PFTII) (Figure 1b). One pulsotype (PFTI) formed by four of the six *S. warneri* evaluated was characterized in one herd (NS) with a three-month interval between samplings (Figure 1c).

Figure 1 – PFGE profiles from ten *Staphylococcus intermedius* (1a), eight *Staphylococcus aureus* (1b) and six *Staphylococcus warneri* (1c) isolated from bovine organic milk from two farms in Sao Paulo State, Brazil, showing the 80% similarity line (red line)



* PFT: pulsed-field type I to III; NS and SC: names of the two organic dairy herds evaluated; I to XIII: number of samplings; AE/AD/PE/PD: mammary quarters (AE=front-left, AD=front-right, PE=back-left, PD=back-right); Inside the parentheses: farm registry number of animals

The persistence of microorganisms in bovine milk may be suspected when the same species is isolated from samples collected consecutively from the same animal (ROBERSON et al., 1996). In fact, our result of PFGE has shown a persistent *S. intermedius* in the farm 2, as one may see in figure 1a, since this species was also

represented by the same digestion pattern. Two *S. aureus* pulsotypes with more than 80% of similarity on PFGE were identified in the two organic herds analyzed in this current study. The organic production of bovine milk was the only common fact between the sampled herds. A recent report described that some specific *S. aureus*

clones were responsible for cases of bovine mastitis in conventional dairy farms (CASTELANI et al., 2013), although data regarding organic dairy farms were still scarce. We also found a common *S. warneri* pulsotype being isolated over a three-month period in the same herd. According to another report, CNS can persist in the mammary gland for months (HOSSEINZADEH; SAEI, 2014).

Although more studies with a bigger number of isolates are needed to find out the epidemiology of *Staphylococcus* spp. in organic production, our results can indicate that there may also be the prevalence of some clones present in this kind of dairy production system. The genotyping of *Staphylococcus* spp. provided data from the two organic Brazilian dairy farms in order to contribute to start to

understand the epidemiology of this pathogen from this kind of dairy system in Brazil.

To the best of our knowledge, the present study describes for the first time in Brazil the presence of enterotoxin genes and *mecA* in *Staphylococcus* spp. isolated from organic dairy farms. The resistance of these isolates associated with their toxigenic potential and their genetic diversity highlighted the public health risk involved in the consumption of raw milk and dairy products contaminated with these bacteria.

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