ijvm.2019.282080.1004991

Iranian Journal of Veterinary Medicine
Volume 13- Issue 04

Original Article

Online ISSN : 2252-0554

RAPD-PCR and Drug Resistance Pattern of *Staphylococcus aureus* Isolates Recovered from Companion and Wild Birds

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Abstract

BACKGROUND: *Staphylococcus aureus* is a highly versatile pathogen of a large number of domestic animals, including avian species. There is limited information about *S. aureus* isolated from companion and wild birds in Iran.

OBJECTIVES: The aim of this study was to determine drug resistance and random-amplified polymorphic DNA-PCR (RAPD-PCR) pattern of *S. aureus* isolated from birds referred to the pet birds' clinic of University of Tehran.

METHODS: During the study period, 53 isolates of *S. aureus* were recovered from companion birds of various species using standard bacteriologic procedures and the respective drug resistance patterns were determined for a panel of 30 antimicrobial agents by agar disk-diffusion method. RAPD-PCR was performed with two different 10-bp oligonucleotide primers in a duplex-PCR procedure.

RESULTS: The findings of this study demonstrated that *S. aureus* resistance to oxacillin, clindamycin and methicillin were 58, 53 and 53%, respectively. The multi-drug resistance (MDR) was found among all isolates. The MDR pattern was variable and ranged from 0 to 17 drugs. In total, all 53 isolates generated 43 different resistance patterns. In RAPD-PCR, five different patterns of A, B, C, D and E were found. Among 53 isolates, 20, 62, 3, 9 and 3% belonged to RAPD patterns of A, B, C, D and E, respectively.

CONCLUSIONS: This study showed the widespread antimicrobial resistance among *S. aureus* isolated from pet birds; in particular, the presence of MRSA isolates. The value of RAPD-PCR for epidemiologic monitoring of *S. aureus* in pet birds also was noticed.

KEYWORDS: Companion birds, drug resistance, molecular epidemiology, RAPD-PCR, *Staphylococcus aureus*

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Received: 2019-06-06
Accepted: 2019-08-10

How to Cite This Article
Bagheri, S., Peighambari, SM., Soltani, M., & Malekan, M. (2019). RAPD-PCR and Drug Resistance Pattern of *Staphylococcus aureus* Isolates Recovered from Companion and Wild Birds. Iranian Journal of Veterinary Medicine, 13(4), 355-364.

Introduction

The genus *Staphylococcus* contains approximately 45 species and 24 subspecies of which *Staphylococcus aureus* is the most important cause of infections (Andreasen, 2013). However, other species are occasionally involved. The infections, sporadic or enzootic, may occur in different avian species with various clinical manifestations including acute septicemia or subacute to chronic arthritis, osteomyelitis and osteitis. Vesicular dermatitis or omphalitis also have been less commonly reported (Andreasen, 2013). Staphylococci, particularly *S. aureus*, are gram positive bacteria that, primarily or secondarily, can infect humans and are of major concern in human medicine. It has been associated with various life-threatening conditions such as pneumonia, osteomyelitis, endocarditis, and septicemia in humans (Watkins et al., 2012). Drug resistance remains a major threat to public health, therefore, understanding the epidemiology of *S. aureus* and its prevalent drug resistance pattern is very important in human and veterinary medicine. Understanding the methicillin resistant *S. aureus* existence in companion animals including pet birds, due to its zoonotic concerns, must be noticed (Simoons-Smit et al., 2000).

In order to type *S. aureus* isolates, various pheno- and genotypic methods have been investigated by different researchers (Luijendijk et al., 1996; Tambic et al., 1999; Butterworth et al., 2001; Lee, 2003; Reinoso et al., 2004). The random-amplified polymorphic DNA-PCR (RAPD-PCR) is a method for *S. aureus* typing and has been designed based on single primer of arbitrary nucleotide sequence, attaching to their possible sites throughout genome, and resulting in a different pattern of amplified DNA segments on the gel. The RAPD-PCR is an appropriate, simple, inexpensive and efficient tool for *S. aureus* typing, and is applicable for further *S. aureus* features recognition.

There is still limited information on the drug resistance and RAPD-PCR pattern of *S. aureus* in birds. Hence, the aim of this study was to genotype 53 *S. aureus* isolates by RAPD-PCR and to determine the drug resistance pattern of *S. aureus* isolates of birds referred to the pet birds' clinic of University of Tehran.

Materials and Methods

Sampling and bacterial isolation

During a 4-month period, various species of companion and wild birds referred to the pet birds' clinic of University of Tehran that were suspected of *Staphylococcus* infection were sampled by swabbing of the related site. Each sample was cultured on 5% defibrinated sheep blood agar and MacConkey agar and observed after 18 and 36 h of incubation at 37.8 ºC. Bacterial growth of all samples were characterized based on morphology, Gram's stain, catalase test, tube coagulase reaction and their ability to ferment mannitol anaerobically (Andreasen, 2013). In total, 53 *S. aureus* isolates were identified, frozen at -70 ºC and kept for future use.

Drug susceptibility test

The susceptibility of the *S. aureus* isolates to a panel of antimicrobial agents was determined by the agar disk diffusion method and the interpretation of results was carried out according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). The antimicrobial agents that were tested and their concentrations (µg) were as follows: oxacillin (1), methicillin (10), clindamycin (2), cefixime (5), penicillin (10), rifampicin

(5), ceftazidime (30), vancomycin (30), kanamycin, (30), erythromycin (15), norfloxacin (10), amoxicillin (25), streptomycin (10), ceftizoxime (30), danofloxacin (10), ofloxacin (5), enrofloxacin (5), ciprofloxacin (5), ampicillin (10), ceftriaxone (30), cefotaxime (30), meropenem (10), cefepime (30), amikacin (30), neomycin (30), trimethoprim/sulfamethoxazole (1.25/23.75), azithromycin (15), gentamicin (10), linco-spectin (15/200), chloramphenicol (30). All antibacterial disks were provided from Padtan Teb Co. (Tehran, Iran) except methicillin (Himedia, Mumbai, India). The ATCC reference strain *S. aureus* ATCC 25923 was used for quality control purposes. The isolates were classified as susceptible, intermediate susceptible, or resistant based on the standard interpretation chart updated according to the CLSI guidelines (CLSI, 2008).

Random-amplified polymorphic DNA (RAPD) analysis

To extract bacterial DNA, each *S. aureus* isolate was individually cultured on Luria-Bertani (LB) agar and incubated overnight at 37 ºC. Template DNA was prepared from each *S. aureus* isolate grown overnight at 37 ºC on LB agar using the MBST Genomic DNA extraction kit (MBST Co., Tehran, Iran). DNA concentration was estimated using spectrophotometry at 260 nm.

Two primers, A (5'-TGCGCCCTTC) and B (5′-GGTGACGCAG) were used for RAPD typing in this study (Butterworth et al., 2001). The primers and other materials used in PCR reaction were provided by SinaClon (Tehran, Iran). Amplifications were carried out in a 25 µl reaction volume containing 2.5 µl 10 x PCR buffer, 1 µl 10 mM dNTP mix, 0.8 µl 50 mM MgCl2, 1 µl (100 ng) of each primer, 0.25 µl (1 unit) of Taq polymerase DNA, 16.45 µl dH2O and approximately 2 µl (200 ng) of template DNA. Negative controls (dH2O instead of template DNA) were included in all PCR reaction sets. Amplification was programmed in a thermocycler (Senso-Quest, Germany) as follows: 94 °C for 105 s followed by 40 cycles of 94 °C for 60 s, 37 °C for 60 s, 72 °C for 180 s, and a final extension at 72 °C for 120 s. The amplified products were detected by gel electrophoresis in 1.5% agarose gel at 100 V for 90 min in 1 x TAE buffer. A commercial DNA ladder, GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific, Germany), was used as the molecular- weight marker in each gel running. Reproducibility of the RAPD patterns was confirmed using triplet runs on separate days but on the same thermocycler.

Results

Drug susceptibility test

The percentages of *S. aureus* isolates that were resistant to the antimicrobial agents were as follows: 58 to oxacillin, 53 to clindamycin, 53 to methicillin, 47 to cefixime, 45 to penicillin, 36 to rifampicin, 34 to ceftazidime, 32 to vancomycin, 32 to kanamycin, 30 to erythromycin, 23 to norfloxacin, 23 to amoxicillin, 23 to streptomycin, 21 to ceftizoxime, 19 to danofloxacin, 19 to ofloxacin, 19 to ampicillin, 17 to ceftriaxone, 17 to cefotaxime, 17 to meropenem, 15 to enrofloxacin, 13 to ciprofloxacin, 13 to amikacin, 13 to neomycin, 11 to trimethoprim/sulfamethoxazole, 11 to cefepime, 11 to azithromycin, 9 to gentamicin, 8 to linco-spectin and 4 to chloramphenicol. Forty-three multi-drug resistance (MDR) patterns were observed among 53 *S. aureus* isolates (Table 1). However, it is noteworthy to mention that the observed MDR patterns were variable, ranging from being resistant to 0 to 17 drugs (Table 2).

Table 1. Drug resistance patterns among 53 companion birds *S. aureus* isolates

#Pattern	Resistant to	No. of isolates (%)
1	V, NOR	3 (5.66)
2	K, CFM, S, P	3 (5.66)
3	V, MET, OX, E, CC, RA, P	Each pattern included two isolates (3.77)
4	DFX, MET, OX, CFM, NFX, OFX, LS, CP, CAZ, NOR	
5	CFM	
6	C	
7	K	
8	K, AN, CRO, MET, OX, CT, CC, N, CFM, CTX, MEN, CAZ	
9	V, DFX, MET, OX, E, RA, NFX, AMX, OFX, S, AM, SXT, P, CP, NOR	
10	CAZ	Each pattern included only one isolate (1.88)
11	V	
12	CC, CXT	
13	CFM, CAZ	
14	OFX, CAZ	
15	CC, SXT, GN	
16	CFM, RA, AM	
17	,MET, OX, CC, AMX	
18	MET, OX, CT, CC	
19	K, CFM, S, AM, P	
20	V, MET, OX, CC, RA, P	
21	V, DFX, E, CTX, NOR	
22	C, OX, CT, CC, AMX, P	
23	OX, CC, CFM, RA, P, CAZ	
24	V, OX, E, CC, RA, AMX, P	
25	V, MET, OX, CC, RA, AMX, P	
26	MET, OX, CC, CFM, AM, MEN, CAZ	
27	E, CC, NFX, OFX, LS, CP, NOR	
28	V, MET, OX, E, CC, RA, AMX, P	
29	V, MET, OX, E, CC, RA, AMX, AM, P	
30	V, AZM, MET, OX, E, CC, RA, AMX, AM, P	
31	MET, OX, CC, CFM, AM, P, MEN, CAZ	
32	K, DFX, MET, OX, CT, CFM, SXT, MEN, CAZ	
33	DFX, MET, OX, CFM, NFX, OFX, CP, CAZ, NOR	

#Pattern	Resistant to	No. of isolates (%)
34	K, AN, AZM, MET, OX, CT, E, CC, N, CFM, GM	Each pattern included only one isolate (1.88)
35	K, AN, AZM, MET, OX, E, CC, N, CFM, CTX, S, MEN, CAZ	
36	CRO, AZM, OX, CT, E, N, CFM, RA, AMX, S, FEP, P, CAZ	
37	K, CRO, MET, CC, CFM, RA, CTX, OFX, FEP, P, CAZ	
38	V, CRO, MET, OX, CT, E, CC, RA, AMX, S, AM, GM, P, NOR	
39	K, CRO, DFX, AZM, MET, OX, E, CC, N, CTX, S, FEP, CAZ	
40	V, K, DFX, MET, C, OX, E, CC, RA, AMX, OFX, SXT, P, LS, CP, NOR	
41	K, AN, CRO, MET, OX, CT, CC, N, CFM, RA, CTX, S, AM, FEP, P, MEN, CAZ	
42	K, AN, CRO, MET, OX, CT, CC, CFM, RA, CTX, AM, S, GM, FEP, P, MEN, CAZ	
43	K, AN, CRO, DFX, MET, OX, CT, CC, CFM, RA, NFX, CTX, OFX, GM, FEP, P, MEN, CP, CAZ, NOR	

OX = Oxacillin, MET = Methicillin, CC = Clindamycin, CFM = Cefixime, P= Penicillin, RA = Rifampicin, CAZ = Ceftazidime, V = Vancomycin, K= Kanamycin, E = Erythromycin, NOR = Norfloxacin, AMX = Amoxicillin, S = Streptomycin, CT = Ceftizoxime, DFA = Danofloxacin, OFX = Ofloxacin, AM = Ampicillin, CRO = Ceftriaxone, CTX = Cefotaxime, MEN = Meropenem, NFX = Enrofloxacin, CP = Ciprofloxacin, AN = Amikacin, N = Neomycin, SXT = Trimethoprim/sulfamethoxazole, FEP = Cefepime, AZM = Azithromycin, GM = Gentamicin, LS = Lincospectin, C = Chloramphenicol

Table 2. Multi-drug resistance level among 53 *S. aureus* isolates from companion birds

No. (%) of resistant isolates	No. of antimicrobial drugs
(100) 53	0
(98) 52	> 1
(86) 46	> 2
(75) 40	> 3
(71) 38	> 4
(60) 32	> 5
(60) 32	> 6
(52) 28	> 7
(41) 22	> 8
(37) 20	> 9
(32) 17	> 10
(26) 14	> 11
(22) 12	> 12
(18) 10	> 13
(13) 7	> 14
(11) 6	> 15
(7) 4	> 16
(7) 4	> 17
(0) 0	> 18

RAPD analysis

Using primers A and B, five different RAPD types (A to E) were observed among 53 isolates (Fig. 1). The RAPD profiles differed in the number of fragments and ranged from 0.2 to 2.5 kb in molecular weight. Among 53 isolates, 11 (20.76%), 33 (62.27%), 2 (3.77%), 5 (9.43%) and 2 (3.77%) showed RAPD patterns of A, B, C, D and E, respectively. The pattern B was the most frequent one which were mostly taken from the cloaca and choanal cleft samples. The pattern D was observed in two isolates, both from canary conjunctivitis. The pattern E was also observed in two isolates obtained from European nightjar (*Caprimulgus europaeus*) and domestic chicken (*Gallus domesticus*) foot pad abscess. All five of the samples taken from African gray parrots (*Psittacus erithacus*) showed pattern A. In this study, four isolates were detected from sulphur-crested cockatoo (*Cacatua galerita*) and sparrow hawk with the pattern C. In the current study, nine isolates were from common myna (*Acridotheres tristis*) and except one isolate from pododermatitis that belonged to pattern A, the rest belonged to pattern B. Additionally, out of six isolates from pododermatitis four isolates were classified as pattern A, whereas all isolates from domesticated ducks (*Anas platyrhyncha*) showed pattern B.

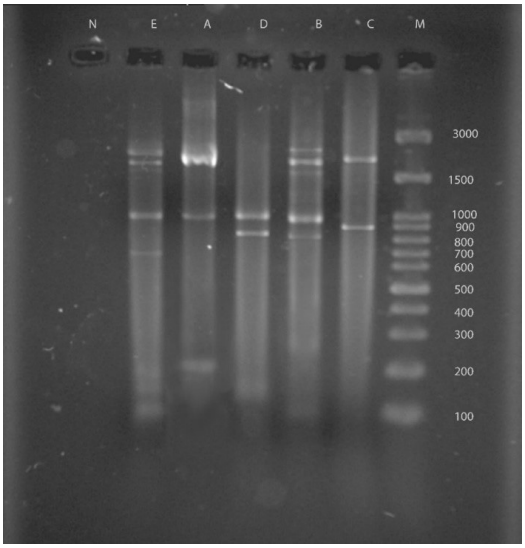

Figure 1. RAPD-PCR sample pattern derived from avian *S. aureus* isolates separated by electrophoresis on 1.5% agarose gel.

Discussion

This study determined the drug resistance patterns and RAPD profiles of 53 *S. aureus* isolates recovered from pet birds. *Staphylococcus aureus* may be the cause of fetal loss, omphalitis, yolk sac inflammation, arthritis, synovitis, septicemia, osteomyelitis, vesicular dermatitis, gangrenous dermatitis and pododermatitis in birds (Andreasen, 2013).

Antimicrobial resistance determination of bacterial isolates recovered from pet birds is of great importance due to the close relationships that exist between pet birds and human beings. The patterns of antimicrobial resistance among *S. aureus* isolates is critical because of the possible presence of methicillin- resistant isolates. In this study, more than 50% of isolates were resistant to methicillin. The methicillin antibiotic is extensively used against human staphylococcal infec-

tions (Harkins et al., 2017). Furthermore, the methicillin-resistant *S. aureus* is frequently reported in human nosocomial infections (Wang and Ruan, 2017; Lounsbury et al., 2019). Therefore, *S. aureus* infection among pet birds is a matter of concern because keeping companion birds is very popular in Iran. The highest resistance rate among isolates of this study was observed in penicillin family. Resistance to clindamycin was also very high (53%) among isolates. Clindamycin is one of the most effective drugs against staphylococcal infections and is being widely administered in companion bird in Iran.

Antimicrobial susceptibility pattern of *S. aureus* isolates originating from avian species have been reported by various researchers. Lee (2003) found 15 PCR mecA-positive MRSA isolates in which 12 were from dairy cows and 3 were from chickens. All isolates were resistant to members of the penicillin family, such as ampicillin, oxacillin, and penicillin (Lee, 2003). In another study, Susa et al. (2014) analyzed the antimicrobial resistance determinants of staphylococcal nasal microbiota in 16 birds of prey and their contents and found that six of the 16 tested animals carried staphylococci (37.5%). The *S. aureus* isolates were penicillin-resistant but methicillin-susceptible. Due to possible contact that may occur among wildlife, domestic animals, humans, insects and even non-living facilities, an increased possibility of interchange of these microorganisms in the different ecosystems may lead to transmission of antimicrobial resistance, including MRSA isolates, to pet birds (Simoons-Smit et al., 2000; El-Mokhtar and Hetta, 2018; Kwok et al., 2018; Abdolmaleki et al., 2019). In a German study, the resistance pheno- and genotypes of 37 MRSA isolates from various sources in four broiler farms were investigated. Except for one farm, isolates from chickens, broiler houses, the farm residences and humans living/working on the same farm were often closely related or indistinguishable. MRSA isolates from the same farm showed apparent identity indicating transmission among broilers, humans and their environment (Wendlandt et al., 2013).

Random amplified polymorphic DNA (RAPD)-PCR has been frequently used to determine molecular epidemiological relatedness of *S. aureus* isolates originated from various sources (Tambic et al., 1999; Butterworth et al., 2001; Lee, 2003; Reinoso et al. 2004). Tambic et al. (1999) in Zagreb detected four RAPD profiles among 36 *S. aureus* isolates from inpatients and staff in a hospital and found that the most common profile involved 15 of 36 tested strains and indicated the RAPD-PCR typing as a useful aid to epidemiological investigations of MRSA (Tambic et al., 1999). In a study in England (Butterworth et al., 2001), 111 *S. aureus* isolates from chickens were typed by RAPD-PCR and four main groups were found based on the observed banding patterns. The predominance of a restricted number of RAPD types in association with pathologies causing lameness was noticed and it was concluded that the putative RAPD groupings may provide a basis for epidemiological studies of *S. aureus* in broiler production systems (Butterworth et al., 2001). Lee (2003) determined molecular epidemiological relatedness of 15 animal MRSA isolates from humans and animals by RAPD patterns, and found a close relationship between the genomes of the six animal MRSA isolates to those of some human MRSA isolates and suggested that those animals may be the possible source of human infections caused by consuming con-

taminated food products. Using RAPD-PCR with three primers, Reinoso et al. (2004) assessed successfully the genetic relationship of *S. aureus* isolates from different hosts, bovine and human in this case (Reinoso et al., 2004). The findings of the present study were comparable to those of other studies. In this study, RAPD-PCR was used for molecular typing of *S. aureus* isolates from companion birds leading to identification of five different RAPD types from A to E, in which the type B was the most frequent one. However, we were not able to exactly correlate a specific RAPD type to a specific pathology.

In conclusion, this study showed that the resistance to antimicrobial agents among *S. aureus* in pet birds is widespread and it is very important to choose the right antimicrobial agent for treatment purposes. The presence of MRSA among *S. aureus* isolates indicates that serious measures need to be taken against such pathogens due to its public health concerns. The present investigation also found the value of RAPD-PCR for epidemiologic monitoring of *S. aureus* in pet birds.

Acknowledgments

This research was supported by grant no. 7508007-6-25 from the Research Council of the University of Tehran.

Conflict of Interest

The authors declare that there is no conflict of interest.

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ijvm.2019.282080.1004991

Iranian Journal of Veterinary Medicine
Abstracts in Persian Language

Online ISSN *2252-0554*

مجله طب دامی ایران، ۱۳۹۷، دوره ۱۳، شماره ۴، ۳۶۴-۳۵۵

الگوی مقاومت دارویی و RAPD-PCR جدایه های *استافیلوکوکوس ارئوس* بدست آمده از پرندگان همراه و وحشی

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(دریافت مقاله: ۱۶ خرداد ماه ۱۳۹۸، پذیرش نهایی: ۱۹ مرداد ماه ۱۳۹۸)

چکیده

زمینه مطالعه: *استافیلوکوکوس اورئوس* به عنوان عامل عفونت‌های استافیلوکوکی در بسیاری از حیوانات از جمله پرندگان شناخته می‌شود. علیرغم اهمیت این پاتوژن در پرندگان زینتی و حیات وحش، تاکنون مطالعه ای در مورد عفونت های استافیلوکوکی در پرندگان زینتی ایران انجام نشده است.

هـــدف: هدف از این مطالعه بررسی الگوی مقاومت دارویی جدایه *استافیلوکوکوس اورئوس* از پرندگان زینتی ارجاعی به کلینیک تخصصی پرندگان دانشکده دامپزشکی دانشگاه تهران و همچنین تعیین الگوی RAPD-PCR این جدایه ها است.

روش کار: طی این مطالعه از پرندگان ارجاعی به کلینیک مذکور نمونه گیری به عمل آمد. پس از تایید با روش های باکتریولوژی، نهایتاً ۵۳ نمونه *استافیلوکوکوس اورئوس* جداسازی گردید. روش استاندارد دیسک دیفوژیون برای تعیین حساسیت جدایه ها به ۳۰ عامل ضد میکروبی مورد استفاده قرار گرفت. همچنین جدایه های *استافیلوکوکوس اورئوس* توسط روش RAPD-PCR با دو جفت پرایمر ۱۰ نوکلئوتیدی تعیین تیپ شدند.

نتایج: مطالعه ی حاضر نشان داد که جدایه‌های *استافیلوکوکوس اورئوس* پرندگان زینتی، بیشترین درصد مقاومت را به اگزاسیلین (۵۸ درصد)، کلیندامایسین (۵۳ درصد) و متی‌سیلین (۵۳ درصد) نشان دادند. در بین جدایه های *استافیلوکوکوس اورئوس*، وقوع مقاومت چندگانه بسیار شایع بود به طوری که آنها حداقل به صفر و حداکثر به ۱۷ دارو مقاوم بودند. همچنین ۴۳ الگوی مقاومت دارویی شناسایی شد. پس از انجام تست RAPD-PCR بر روی ۵۳ نمونه *استافیلوکوکوس اورئوس* پرندگان، ۵ الگو بدست آمد. از مجموع ۵۳ نمونه ای که مورد آزمایش قرار گرفته بودند ۲۰ درصد نمونه ها الگوی A، ۶۲ درصد الگوی B، ۳ درصد الگوی C، ۹ درصد الگوی D و ۳ درصد الگوی E را نشان دادند.

نتیجه گیری نهایی: نتایج مطالعه حاضر مقاومت آنتی بیوتیکی گسترده را در بین جدایه های *استافیلوکوکوس اورئوس* در پرندگان زینتی به ویژه حضور جدایه های MRSA را نشان داد. همچنین ارزش روش RAPD-PCR برای پایش اپیدمیولوژیک *استافیلوکوکوس اورئوس* در پرندگان زینتی مورد تایید قرار گرفت.

واژه‌های کلیدی:

استافیلوکوکوس اورئوس، پرندگان زینتی، مقاومت دارویی، RAPD-PCR، اپیدمیولوژی ملکولی

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