Species diversity and antibiotic resistance properties of *Staphylococcus* of farm animal origin in Nkonkobe Municipality, South Africa

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Abstract The occurrence and antibiotic susceptibility profile of *Staphylococcus* isolates of healthy farm animal origin in Nkonkobe Municipality as well as the prevalence of putative antibiotic resistance genes were investigated using phenotypic and molecular methods. A total of 120 *Staphylococcus* isolates were isolated from 150 animal samples and consisted of *Staphylococcus haemolyticus* (30 %) and *Staphylococcus aureus* (23.3 %) from pig, *Staphylococcus capitis* (15 %) from goat, *S. haemolyticus* (5 %) and *Staphylococcus xylosus* (15 %) from cattle, and other staphylococci (11.7 %) from dead chicken and pigs. Besides this, the presence of these isolates was observed from the animal dung, showing that the organisms are shed to the environment. About 23.3 % of these isolates were coagulase-positive and 76.7 % were coagulase-negative *Staphylococcus*. Between 75 and 100 % of the isolates were resistant to penicillin G, tetracycline, sulfamethoxazole, and nalidixic acid; about 38 % were methicillin-resistant staphylococci, including 12.6 % methicillin-resistant *S. aureus* from pigs. In total, 12 % of all isolates were vancomycin resistant. Also, 12 % of the isolates were erythromycin resistant, while 40.2 % were resistant to ceftazidime. Only the genes *mecA* and *mphC* could be confirmed, whereas the genes *vanA*, *vanB*, *ermA*, *ermB*, and *ermC* could not be detected. The high phenotypic antibiotic resistance and the presence of some associated resistance genes is a potential threat to public health and suggest the animals to be important reservoirs of antibiotic resistance determinants in the environment.

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Introduction

The role of commensals in the spread of resistance among bacteria around the globe cannot be overemphasized (Levy 1986; Summers 2002). They have been fingered as the possible sources of antibiotic resistance genes which are now rampant among many systemic bacterial pathogens of suppurative and non-suppurative infections (Saha et al. 2007; Kitara et al. 2011). The prior exposure of the commensals to antibiotics during chemotherapy for other infections in their hosts might be a selective force for antibiotic resistance (Cohen 1992), especially in human and in animal husbandry, where antibiotics are used in large quantities (Moulin 2001; Harada and Asai 2010). A number of glycopeptides being used as growth promoters in animal husbandry have been reported as inducers of antibiotic resistance in the animal body flora (Perrier-Gros-Claude et al. 1998; Hammerum et al. 2010). The subsequent emergence of resistance has heightened bacterial colonization and/or invasion of the animals, with reduced response to control leading to their persistence as contaminants in animal products such as unpasteurized milk (Kaur and Pathania 2010; Ogbodo et al. 2011). Zoonotic transfer of such difficult-to-treat bacterial species is becoming a worrisome trend, as it increases the disease burden in sub-Saharan African countries, leads to higher cost of procuring treatment options, and increases infant mortality (WHO 2011)

Staphylococcus spp. are among the important commensals of farm animals that often bear resistance genes. It is worthy to recall that the emergence of methicillin resistance was reported in *S. aureus* following the antibiotic introduction in the 1960s (Grundmann et al. 2006). This attribute was first reported in hospitals but later in the community. Today, the methicillin-resistant *S. aureus* (MRSA) is still a threat to global health and wellness (Van Loo et al. 2007). Other

previously known commensal *Staphylococcus* spp. now constitute threats to global health. Some coagulase-negative staphylococci (CoNS) that colonize animal skin and mucous membrane as flora (Kloos and Bannerman 1994) are now implicated in skin and soft tissue infection of man (Shittu et al. 2004), bacteriemia, and septicemia (Komolafe and Adegoke 2008; D'mello et al. 2008). *Staphylococcus haemolyticus*, *Staphylococcus capitis*, and *Staphylococcus xylosus* which are commensals of farm animals can also cause opportunistic infection(s) in other cases (Thorberg 2008).

Weese et al. (2005) and Walther et al. (2008) reported the notable presence of MRSA in animals. The MRSA has been increasingly reported in cattle, goat, sheep, cats, camel, and dogs, and have been examined for resistance to such vital antibiotics by Walther et al. (2008) and Saleha et al. (2010). While other animals also harbor MRSA alike, pigs are vital examples, known to harbor typical MRSA which spreads rapidly among the entire swine (Cuny et al. 2010; Van den Broek et al. 2009). These strains have been found among piggery workers, suggesting a zoonotic transfer (Denis et al. 2009; Van den Broek et al. 2009).

While the MRSA strains in North America (Smith et al. 2009) and Singapore (Sergio et al. 2007) are well documented, information on their counterparts in sub-Saharan Africa especially in the Eastern Cape Province of South Africa is very scarce; ditto for information on the antibiotic resistance properties of commensal bacteria of animal origin. In this study, the prevalence of staphylococci of farm animal origin in the Nkonkobe Municipality, Eastern Cape Province, South Africa as well as their phenotypic and genotypic resistance properties were investigated.

Materials and methods

Study location

The study location is the Nkonkobe Municipality, a highly populated domain in the Eastern Cape Province, South Africa, with a population of about 128,658 on a 3,724 km^2 of land. The *Staphylococcus* spp. of farm animal origin were isolated from nasal, mouth wash, and ear swabs of pigs (including the piglets), cattle, goats, and chickens from various animal farms within the municipality. Some samples of the animal dung were also analyzed to see whether the isolates were shed to the environment in the animal dung.

Isolation and preliminary identification of *Staphylococcus* species

Preliminary isolation of *Staphylococcus* spp. of farm animal origin was initiated by inoculating the swabs directly in

nutrient broth (CM0001B, Oxoid, England) and incubated at 37 °C for 24 h (Cheesebrough 2006). Thereafter, a loopful of inoculum was transferred from each turbid tube to mannitol salt agar (Oxoid, England) and incubated also for 24 h at 37 °C (Cheesebrough 2006). At the end of the incubation period, distinct colonies were purified and subjected to gram-staining, catalase test, coagulase test, and lysostaphin susceptibility test. The *Staphylococci* were preliminarily speciated using the analytical profile index (API) Staph (BioMerieux). The results were read following the incubation of inoculated strips between 18 and 24 h.

Genus- and specie-specific identification of *Staphylococcus* species

All isolates identified earlier using the API Staph kit were confirmed by genus-specific and species-specific polymerase chain reactions (PCR) using the primers listed in Table 1. The PCR conditions employed for the genus level identification include 3 min at 96 °C, 40 cycles of 30 s at 95 °C, 60 s at 55 °C, 30 s at 72 °C, and a final extension of 3 min at 72 °C (Martineau et al. 2001). *S. aureus* ATCC 25923 was used as the positive control, while nuclease-free water was used as the negative control. The amplicons were electrophoresed on 2.5 % agarose gel stained with ethidium bromide and visualized under UV light. Species-specific identification was done by multiplex PCR targeting *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *S. xylosus*, and *S. aureus* (at the amplicon size in Table 1), and the PCR conditions include 15 min at 4 °C, 3 min at 94 °C, then 40 cycles of 1 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and a final hold of 3 min at 72 °C (Corbière et al. 2004). The amplicons were electrophoresed in 1× TBE buffer on 2 % agarose gel stained with ethidium bromide and visualized under UV light. Other species were identified by simplex PCR with the conditions which include a denaturation step 10 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 30 s at the appropriate annealing temperature of 59, 50, and 60 °C for *S. capitis*, *S. haemolyticus*, and *Staphylococcus warneri*, respectively, and 30 s at 72 °C were employed (Iwase et al. 2007).

Phenotypic antibiotic susceptibility profile

The standard agar disk diffusion technique was employed to determine the antibiotic susceptibility pattern of the isolates, and this was performed in accordance with standards described by Cheesebrough (2006) and Clinical and Laboratory Standards Institute (CLSI) (2008). The antibiotics used include penicillin G (11 U), ampicillin (10 μg), oxytetracycline (10 μg), minocycline (10 μg), streptomycin (10 μg), cotrimoxazole (25 μg), cefotaxime (10 μg), erythromycin (10 μg), gentamicin (10 μg), clindamycin (2 μg), ceftriaxone (30 μg), oxacillin (5 μg), ceftriaxone (30 μg), ceftazidime

Table 1 Genus and species-specific identification primers used

Organisms		Primers	Primer sequence (5′-3′)	Size (bp)	References
Staphylococci	Genus	TStaG422 TStag765	GGC CGT GTT GAA CGT GGT CAAATCA TIA CCA TTT CAG TAC CTT CTG GTA	370	Martineau et al. (2001)
	S. capitis	Scap F Scap R	GCTAATTTAGATAGCGTACCTTCA CAGATCCAAAGCGTGCA	208	Iwase et al. 2007
	S. haemolyticus	ShaeF ShaeR	GTTGAGGGAACAGAT CAGCTGTTTGAATATCTT	85	
	S. warneri	SwarF SwarR	TGTAGCTAACTTAGATAGTGTTCCTTCT CCGCCACCGTTATTTCTT	63	
	S. xylosus	Xyl F Xyl R	AACGCGCAACGTGATAAAATTAATG AACGCGCAACAGCAATTACG	539	Morot-Bizot et al. (2004)
	S. saprophyticus	Sap1 Sap2	TCAAAAAGTTTTCTAAAAAATTTAC ACGGGCGTCCACAAAATCAATAGGA	221	
	S. aureus	Sa442-1 Sa442-2	AATCTTTGTCGGTACACGATATTCTTCACG CGTAATGAGATTTCAGTAGATAATACAACA	1,108	
	S. epidermidis	Se705-1 Se705-2	ATCAAAAAGTTGGCGAACCTTTTCA AAAAGAGCGTGGAGAAAAGTATCA	1,124	

(30 μg), vancomycin (5 μg), cephalothin (25 μg), imipenem (10 μg), meropenem (10 μg), ofloxacin (5 μg), levofloxacin (5 μg), and ciprofloxacin (5 μg). *S. aureus* ATCC 25923 was used as quality control. The results were interpreted according to the CLSI standards (NCCLS 1999; CLSI 2008).

Multiple antibiotic resistance index

The multiple antibiotic resistance index (MARI) was calculated as the ratio of the number of the antibiotics to which resistance occurred by the isolates (a) to the total number of antibiotics to which the isolates were tested (b) (Krumperman 1983).

$$MARI = a/b$$

All the antibiotics used were also considered for MARI determinations as organisms' exhibit unequal profile to antibiotics within the same class.

PCR detection of antibiotic resistance genes

Table 2 summarized the list of primers used for the PCR detection of *ermA*, *ermB*, *ermC*, *msrA*, and *mphC* genes following the protocol of Sauer et al. (2008) with slight modifications. The genomic DNA was extracted using PrepMan Ultra DNA extraction kit (Applied Biosystem). PCR cycles involve an initial denaturation step of 94 °C for 5 min followed by 30 amplification cycles including denaturation at 94 °C for 60 s, annealing at 51 °C for *ermA*, *ermB*, *ermC*, or 55 °C for *msrA*, *mphC* for 60 s, and extension at 72 °C for 60 s. A final extension at 72 °C for 5 min in 1 cycle

then ended the PCR. For *mecA*, *vanA*, and *vanB* genes, PCR conditions include an initial denaturation step at 94 °C for 5 min, which will be followed by 10 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 45 s) and another 25 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s, and extension at 72 °C for 2 min), ending with a final extension step at 72 °C for 10 min.

Statistical analysis

The observed variables were converted into easily interpretable data. The significance of these data was determined using chi-square. A p value ≤0.05 was regarded as being statistically significant, while p values ≥0.05 was interpreted as being statistically nonsignificant (Dahiru 2008)

Results

Following phenotypic and molecular identification, 120 staphylococcal isolates of farm animal origin were recovered. The analytic profile index results showed well over 95 % agreement for genus-based identification, when compared with PCR-based genus-specific identification. Species-specific PCRs revealed the identity of the following staphylococcal species: *S. xylosus* (15 %), *S. aureus* (23.3 %), *S. haemolyticus* (35 %), *S. capitis* (15 %), and other *Staphylococcus* spp. (11.7 %) (Table 3).

For clarity, Table 3 specifically showed the recovery of *Staphylococcus* spp. with respect to their animal source(s). Sixty-four (55.6 %) of the isolates were recovered from pigs,

Table 2 Primers used to assess the antibiotic resistance genes

Genes	Primer	Sequence (5′→3′)	Amplicon size (bp)	References
ermA	erm(A)-1	GCGGTAAACCCCTCTGAG	434	Werckenthin and Schwarz (2000)
	erm(A)-2	GCCTGTCGGAATTGG		
ermB	erm(B)-1	CATTTAACGACGAAACTGGC	425	Jensen et al. (1999)
	erm(B)-2	GGAACATCTGTGGTATGGCG		
ermC	erm(C)-1	ATCTTTGAAATCGGCTCAGG	295	Jensen et al. (1999)
	erm(C)-2	CAAACCCGTATTCCACGATT		
msrA	msr(A)-1	GCAAATGGTGTAGGTAAGACAACT	400	Wondrack et al. (1996)
	msr(A)-2	ATCATGTGATGTAAACAAAAT		
mphC	mph (C)-1	GAGACTACCAAGAAGACCTGACG	722	Lüthje and Schwarz (2006)
	mph (C)-2	CATACGCCGATTCTCCTGAT		
mecA	mecA1	GTAGAAATGACTGAACGTCCGATAA	310	Geha et al. (1994)
	mecA2	CCAATTCCACATTGTTTCGGTCTAA		
vanA	van A1	GGGAAAACGACAATTGC	732	Dutka-Malen et al. (1995)
	van A2	GTACAATGCGGCCGTTA		
vanB	van B1	GTGCTGCGAGATACCACAGA	1145	Ramos-Trujillo et al. (2003)
	van B2	CGAACACCATGCAACATTTC		

18 (15.7 %) from goats, and 24 (20.9 %) from cattle. These percentages refer to the quantity per total *Staphylococcus* spp. isolated. Twenty-eight (23.3 %) of the *Staphylococcus* spp. were coagulase-positive, while the remaining were coagulase-negative.

The results of antimicrobial susceptibility testing are shown in Table 4. Resistances of 40.2 % to ceftazidime, 75 % to penicillin G, 83.3 % to tetracycline, 100 % to nalidixic acid, and sulfamethoxazole were observed. Also, 38 % of the *Staphylococcus* spp. were resistant to oxacillin, while 12 % showed phenotypic resistance to vancomycin. The presence of the *mecA* genes was observed among the methicillin-resistant *Staphylococcus* spp. No vancomycin resistance genes (*vanA* and *vanB*) were detected in these organisms. Also, 12 % of the bacteria were resistant to erythromycin, while 40.2 % were resistant to ceftazidime. Over 68.4 % of the isolates were classified as multiresistant according to the MARI >2 (Fig. 1).

Discussion

The ability of staphylococci to survive in unfavorable osmotic conditions, pressure, and slightly elevated temperature supports their survival of staphylococci on animals (LeLoir et al. 2003). We were particularly interested in those species that can affect humans. In total, 30 % of the *Staphylococci* from pigs were *S. haemolyticus* and 23.3 % *S. aureus*. In our study, *S. aureus* has been only detected among the porcine isolates, which is in accordance with De Neeling et al. (2007). *S. haemolyticus* and some unidentified staphylococcal isolates were also observed from pig sources. *S. capitis* were isolated from goats only. *S. capitis* is a known flora of human scalp and skin, though it is also a frequently observed etiology of endocarditis (Van Der Zwet et al. 2002; Iwase et al. 2007; D'mello et al. 2008). About 5 to15% of the total staphylococcal isolates from the animal sources were *S. haemolyticus* and *S. xylosus*, respectively, and they were isolated from cattle.

**Table 3** Prevalence/frequency of occurrence of the *Staphylococcus* spp. with respect to sample source

S/N	Animal source(s)	Species	No (%) of occurrence	Antibiotics of interest to which resistance occurred
1	Pig	*S. haemolyticus*	36 (30.0)	Methicillin/oxacillin (38 %), vancomycin (12 %)
		S. aureus	28 (23.3)	
2	Goat	*S. capitis*	18 (15.0)	
3	Cattle	*S. haemolyticus*	6 (5.0)	
		S. xylosus	18 (15.0)	
4	Dead chicken, pig, cattle dung	Staphylococcal species (unspeciated)	14 (11.7)	
	Total		120 (100)	

Table 4 Antibiotic susceptibility profile of the *Staphylococcus* spp

Antibiotic	S (%)	I (%)	R (%)
Penicillin G	25.0	0	75.0
Meropenem	97.7	0	2.3
Vancomycin	87.0	1.0	12.0
Cefotaxime	78.0	9.0	13.0
Ceftazidime	26.5	33.3	40.2
Oxacillin	44.0	18.0	38.0
Minocycline	73.0	11.0	16.0
Tetracycline	16.7	0	83.3
Erythromycin	73.0	15.0	12.0
Clindamycin	53.0	31.0	16.0
Chloramphenicol	91.7	8.3	0
Nalidixic Acid	0	0	100.0
Ciprofloxacin	74.0	23.0	3.0
Ofloxacin	83.0	12.0	5.0
Levofloxacin	98.0	0	2.0

S susceptible, *I* intermediate, *R* resistance

The total occurrences of *S. haemolyticus* observed in this study agrees with earlier reports on animal and animal products as reservoir of bacterial pathogens (Bagcigil et al. 2007; Schlegelova et al. 2008). Since there are at least 40 recognized *Staphylococcus* spp. (Trulzsch et al. 2002; Bannerman 2003), assaying for all of them may not be feasible in this kind of study (Deurenberg and Stobberingh 2008). Hence, 11.7 % of the *Staphylococcus* spp. identified to genus level and recovered from pigs and chicken could not be speciated into any of the target species listed in Table 3. The recovery of more CoNS (76.7 %) corroborates previous reports (Gortel et al. 1999; Kania et al. 2004). The elaboration of staphylocoagulase, as observed in this study, has been described as a very important factor in determining the inherent pathogenicity of bacteria also in commensal flora (Fairbrother 2005).
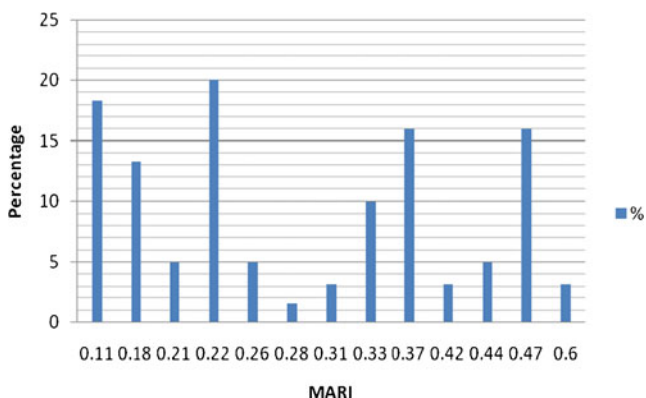
Fig. 1 Multiple antibiotic-resistant index and the percentage of isolates involved *NOTE: MARI ≥0.2 depict the source as high-risk source

The observed resistance patterns to some of the conventional antibiotics which are usually frequently prescribed in the study area are alarming because of the high resistance rates among nonclinical isolates. This further reaffirms the critical role of commensals in public health. The observed high level (75–100 %) of resistance to sulfamethoxazole, nalidixic acid, tetracycline, and improved penicillin G, all of which are broad-spectrum antibiotics, might be due to consumption of antimicrobials (Moulin 2001) as growth promoters (Perrier-Gros-Claude et al. 1998), as extensively practiced in the study area. Of the 38 % resistance to methicillin/oxacillin observed, 12.6 % was *S. aureus* from pig. Though oxacillin is more stable than methicillin during storage, laboratory diagnosis of methicillin resistance depends on the testing of oxacillin, and methicillin/oxacillin-resistant staphylococcal isolates are supposed to be reported as being resistant to β-lactam antibiotics (CLSI 2008). Vancomycin used to be the last antibiotic for treating infections caused by such resistant isolates (Fitzgerald et al. 2001; Boucher et al. 2010). In fact, it was a drug in the last line of defense (Bhalakia and Morris 2005). In this study, 12 % of the staphylococcal isolates were vancomycin-resistant and were recovered from all animal species investigated in this study. Unfortunately, there is usually a close link between the resistance to vancomycin and to other extended spectrum beta-lactam drugs like meropenem and imipenem, with a tendency to worsen the difficulty in the choice of therapeutic options (Chang et al. 2003; Boucher et al. 2010). Tenover and Goering (2009) also reported the presence of community-based MRSA, just as Bhalakia and Morris (2005) also reported the presence of plasmid-mediated vancomycin resistance in fomite. The MAR index of >0.2 observed in this study suggests that the isolates emerged from high-risk sources (Suresh et al. 2000).

The observed phenotypic methicillin/oxacillin resistance in this study was confirmed by the detection of the *mecA* genes. The *mecA* gene confers resistance against β-lactamase-stable penicillin and virtually all other β-lactam antibiotics (Pinho et al. 2001; Weese et al. 2005). The *mecA* gene codes for an alternative penicillin binding protein PBP2a which has low affinity for β-lactam antibiotics (Pinho et al. 2001). The observed absence of *mecA* gene among few methicillin-resistant *Staphylococcus* spp. in this study corroborates the observation of Montanari et al. (1990) that the presence of underlining factors for methicillin resistance apart from *mecA* genes. The presence of this gene in the commensal organisms might render them difficult to control, given the opportunity to zoonotically infect immunocompromised individuals. This gene is usually housed in a large mobile genetic element known as staphylococcal cassette chromosome *mec* (SCC*mec*) (Weese et al. 2005). Meanwhile, at least 11 different SCC*mec* types have been described, and some of them are found in humans (Kriegeskorte et al. 2012). This has been

described as hospital-associated and/or community-associated MRSA (Otter and French 2010). We, therefore, proposed that the observed MRSA in the pig and methicillin-resistant *S. haemolyticus* could also have been transferred from human sources to the animals, as they are also possible colonizers of human hosts. Transfer to these animals from man through an intermediate animal is also a possibility. However, the presence of the *mecA* gene gives a genetic basis for resistance of the *Staphylococcus* spp. to methicillin and other lower beta-lactam antibiotics (Duquette and Nuttall 2004), even if phenotypic susceptibility to beta-lactam is seemingly observed.

Lower resistance (12 %) of the *Staphylococcus* spp. to erythromycin was observed than to the third generation cephalosporin, ceftazidime (40.2 %). This confirms the earlier report by Essack (2001) that some lower class β-lactam antibiotics showed better activities to gram-positive bacteria than third generation cephalosporin. The detection of *mphC* gene justifies the phenotypic resistance to erythromycin and serves as representative of such genes among commensals in the study area. Meanwhile, the wide range of multiple antibiotic resistance indices showed a divergence between the static-use (in situation where a fixed antibiotic regimen is used) and the adaptive-use (in using varieties or wide range of antibiotics due to observed low performance of the earlier one(s)), which may imply consistent use of various antibiotics in these farms on the animal, to achieve a non-chemotherapeutic advantage (Laxminarayan and Klugman 2011). This implies that the organisms might have developed resistance over a period of exposure without medical prescription. An observation of MAR index >0.2 means that the isolate source is high risk, which is a source where antibiotics are in constant abuse, and the act is bringing about high selective pressure (Suresh et al. 2000).

On the animal impact on the environment, the presence of these isolates in the animal dung showed that the *mecA*-positive isolates are released to the environment by these organisms. This corroborates the observation of Alexander et al. (2011).

Therefore, this exposure of the animal bacterial flora to antibiotics appears to be an encouraging emergence of resistance across a wide range of antibiotics (Barbosa and Levy 2000). It is, therefore, important to control the misuse or any other non-therapeutic use of antibiotics. This study is, therefore, important to the agriculture sector in South Africa as animal health is involved. It is also important to the health sector due to the possibility of zoonotic infections, if care is not taken, even if the organism in the animals has its original source from previous human contact. Piggery workers should be diligently hygienic as the animal is a consistent source of MRSA. Regular PCR-based assessment of MRSA prevalence in various aspects of natural life and hospitals is hereby advocated to bring about appropriate control strategies and to reduce the present scourge of MRSA in multidrug resistant outbreaks.

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