Etiology, antimicrobial susceptibility profile of *Staphylococcus* spp. and risk factors associated with bovine mastitis in the states of Bahia and Pernambuco[1]

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The purpose of this paper was to study the etiology of mastitis, determine the antimicrobial susceptibility profile of *Staphylococcus* spp. and to identify the risk factors associated with infection in dairy cows in the states of Bahia and Pernambuco, Brazil. From the 2,064 milk samples analyzed, 2.6% were associated with cases of clinical mastitis and 28.2% with subclinical mastitis. In the microbiological culture, *Staphylococcus* spp. (49.1%) and *Corynebacterium* spp. (35.3%) were the main agents found, followed by *Prototheca* spp. (4.6%) and Gram negative bacilli (3.6%). In the antimicrobial susceptibility testing, all 218 *Staphylococcus* spp. were susceptible to rifampicin and the least effective drug was amoxicillin (32.6%). Multidrug resistance to three or more drugs was observed in 65.6% of *Staphylococcus* spp. The risk factors identified for mastitis were the extensive production system, not providing feed supplements, teat drying process, not disinfecting the teats before and after milking, and inadequate hygiene habits of the milking workers. The presence of multiresistant isolates in bovine milk demonstrates the importance of the choice and appropriate use of antimicrobial agents. Prophylactic and control measures, including teat antisepsis and best practices for achieving hygienic milking should be established in order to prevent new cases of the disease in herds.

INDEX TERMS: *Staphylococcus* spp., multidrug resistance, mammary gland, risk factors.

RESUMO.- [**Etiologia, perfil de sensibilidade dos *Staphylococcus* spp. aos antimicrobianos e fatores de risco associados à mastite bovina nos estados da Bahia e Pernambuco.**] Objetivou-se estudar a etiologia da mastite, determinar o perfil de sensibilidade dos *Staphylococcus* spp. aos antimicrobianos e identificar os fatores de risco associados à infecção em vacas leiteiras nos estados da Bahia e Pernambuco. Das 2.064 amostras de leite analisadas, 2,6% estavam associadas a casos de mastite clínica e 28,2% à mastite subclínica. No exame microbiológico, *Staphylococcus* spp. (49,1%) e *Corynebacterium* spp. (35,3%) foram os principais agentes isolados, seguidos de *Prototheca* spp. (4,6%) e bacilos Gram negativos (3,6%). No teste de sensibilidade aos antimicrobianos, todos os 218 *Staphylococcus* spp. apresentaram-se sensíveis à rifampicina e a droga menos eficaz foi a amoxicilina (32,6%). A resistência simultânea a três ou mais drogas foi observada em 65,6% dos *Staphylococcus* spp. Os fatores de risco identificados para a mastite foram o sistema de criação extensivo, não realização de suplementação alimentar, processo de seca-

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gem dos tetos, não realização de desinfecção dos tetos antes e após a ordenha e hábitos higiênicos inadequados dos ordenhadores. A presença de isolados multirresistentes no leite bovino demonstra a importância da escolha e da utilização adequada de antimicrobianos. Medidas de controle e profilaxia, incluindo a antissepsia dos tetos e boas práticas para a obtenção de ordenha higiênica devem ser estabelecidas, com o intuito de prevenir novos casos da doença nos rebanhos.

TERMOS DE INDEXAÇÃO: *Staphylococcus* spp., multirresistência, glândula mamária, fatores de risco.

INTRODUCTION

Brazil occupies sixth position in milk production worldwide. The Northeast region is responsible for approximately 10% of all bovine milk produced in the country, with production especially in the states of Bahia, Pernambuco and Ceara (IBGE 2011). In these locations, a large part of dairy activity is directed to the subsistence of small rural properties, playing an important economic and social role (Vilela et al. 2002). From the technological point of view, the quality of the raw material is one of the greatest obstacles to the development and consolidation of the dairy industry in Brazil (Guimarães & Langoni 2009).

Bovine mastitis is associated with reduction in milk production and causes changes in milk composition, and it is recognized as one of the main illnesses that affect the profitability of dairy farms (Bradley 2002). In addition, the disease presents a public health risk through the possibility of transmission of pathogenic microorganisms, toxins or antimicrobial residues through the milk (Fagundes & Oliveira 2004). Among the agents of contagious origin, *Staphylococcus* spp. are the bacteria most frequently isolated from clinical and subclinical cases (Taponen & Pyörälä 2009, Mota et al. 2012). Other microorganisms including *Corynebacterium bovis*, *Streptococcus* spp., *Escherichia coli*, *Klebsiella pneumoniae*, algae and yeasts are also reported in the etiology of intramammary infections (Corbellini et al. 2001, Langoni et al. 2011).

In Brazil there are few studies on the risk factors associated with mastitis (Souza et al. 2005, Oliveira et al. 2012), which are important for knowledge of effective measures in a prevention and control program for the disease (Coentrão et al. 2008). In addition to the adoption of hygiene--health management practices, antibiotic therapy may be an effective strategy in reduction of mastitis rates in a herd (Barlow 2011).

Considering the high prevalence of the illness in herds and the losses to the milk production chain, the purpose of this paper was to study the etiology of mastitis, determine the antimicrobial susceptibility profile of *Staphylococcus* spp. and identify the risk factors associated with infection in dairy cows in the states of Bahia and Pernambuco, Brazil.

MATERIALS AND METHODS

This study was based on analysis of 2064 bovine milk samples originating from 525 lactating cows from eight properties (coded from A to H) in the states of Bahia (n=426) and Pernambuco (n=1638), with seven located in the Lower Middle São Francisco Valley region and one in the Agreste of Pernambuco. At the time of visit to the properties, a questionnaire was applied consisting of objective questions to herd managers to obtain data regarding general characteristics of the property, animal management, hygiene management practices during milking and the milking workers profile. Which antimicrobial drugs were used in treatment of mastitis and other diseases in the herds under study were also checked.

Initially, physical examination of the mammary gland and milk from the animals was made and then the California Mastitis Test (CMT) was performed (Schalm & Noorlander 1957). Samples for microbiological examination were collected after washing the teats with soap and water, drying with paper towel and undertaking antisepsis of the ostium of the teats with alcohol at 70%. Regardless of the reaction of the milk samples in the CMT, on average, 5 mL of milk from each mammary quarter for each animal was collected in labeled sterile containers.

Ten microliters aliquots of cows' milk were streaked in 5% sheep blood agar and then the plates were incubated at 37°C for 48 hours. Microorganisms were identified by means of morphological (coloring, size, presence or absence of hemolysis of the colonies), tinctorial (Gram staining) and biochemical characteristics, according to Quinn et al. (1994). The samples that showed isolation of three or more different microorganisms were considered contaminated (National Mastitis Council 1999).

The *in vitro* susceptibility profile was determined in 218 isolates of *Staphylococcus* spp. by the disk diffusion method (Bauer et al. 1966). Disks with the following antimicrobial agents: amoxicillin (10µg), ampicillin (10µg), cephalexin (30µg), ciprofloxacin (5µg), doxycycline (30µg), enrofloxacin (5µg), erythromycin (15µg), streptomycin (10µg), gentamicin (10µg), lincomycin (2µg), oxacillin (1µg), penicillin (10µg), rifampicin (5µg), trimethoprim-sulfamethoxazole (25µg) and tetracycline (30µg). Results were interpreted through reading of the inhibition zones observed (CLSI 2008). For quality control of the technique and of the disks used, *Staphylococcus aureus* ATCC 25923 was used. Isolates that showed resistance to three or more drugs tested were considered multiresistant.

To identify the risk factors associated with infection, univariate analysis of the variables of interest was performed by the Pearson chi-square test or Fisher's exact test, when necessary. After that, logistic regression was performed considering the mi-

Table 1. Microorganisms isolated from clinical and subclinical mastitis in dairy cows in the states of Bahia and Pernambuco

Microorganisms	Subclinical mastitis		Clinical mastitis	Total	%
	CMT[a] -	CMT +			
Staphylococcus spp.	124	132	3	259	49.1
Corynebacterium spp.	104	80	2	186	35.3
Prototheca spp.	0	14	10	24	4.6
Gram negatives	11	7	1	19	3.6
Streptococcus spp.	4	4	3	11	2.1
Staphylococcus spp. + *Corynebacterium* spp.	3	6	1	10	1.9
Bacillus spp.	9	0	0	9	1.7
Enterococcus spp.	4	1	0	5	0.9
Yeast	0	1	1	2	0.4
Staphylococcus spp. + Gram negatives	0	0	1	1	0.2
Staphylococcus spp. + *Bacillus* spp.	1	0	0	1	0.2
TOTAL	260	245	22	527	100.0

[a] California Mastitis Test.

crobiological examination (positive or negative) as the dependent variable. The independent or explanatory variables considered in the model were those that showed statistical significance (<0.2). This probability was stipulated so that possible risk factors of the event would not be excluded from analysis (Hosmer & Lemeshow 1989). Statistical calculations were carried out through use of the program Epi Info, version 3.5.1, Centers for Disease Control and Prevention (CDC).

Table 2. Multidrug resistance profile of *Staphylococcus* spp. isolates from dairy cows on farms in Bahia and Pernambuco to three or more antimicrobial drugs

Properties	Multidrug resistance profile		Total MR[a]
	3-4 drugs	5-7 drugs	
Bahia			
A (n=5)	5	0	5 (100.0%)
B (n=85)	42	19	61 (71.7%)
Pernambuco			
C (n=3)	1	0	1 (33.4%)
D (n=9)	8	1	9 (100.0%)
E (n=10)	2	1	3 (30.0%)
F (n=11)	3	3	6 (54.5%)
G (n=42)	35	3	38 (90.5%)
H (n=53)	19	1	20 (37.7%)
TOTAL	115 (80.4%)	28 (19.6%)	143 (100.0%)

[a] Total of multiresistant isolates.

Table 3. Univariate analysis of factors associated or not associated with bovine mastitis according to production characteristics on rural properties in Bahia and Pernambuco

Variable	N	Microbiological (Positive)	Univariate analysis OR[a] (IC[b] 95%)	p value
Breed characteristic				
Pure	334	84 (25.1%)	0.97 (0.73–1.28)	0.460
Mixed	1730	443 (25.6%)		
Animal husbandry system				
Intensive	1566	282 (18.0%)	-	0.000*
Extensive	39	25 (64.1%)	8.13 (4.00–17.12)	
Semi-intensive	459	220 (47.9%)	0.52 (0.24–1.06)	
Water source				
Standing water	1730	443 (25.6%)	-	0.836
Flowing water	325	81 (24.9%)	0.96 (0.72–1.28)	
Standing + Flowing	9	3 (33.3%)	1.51 (0.24–7.24)	
Feed supplementation				
Yes	2024	507 (25.0%)	0.33 (0.17–0.63)	0.000*
No	40	20 (50.0%)		
Milk production/day				
Less than 300 liters	130	67 (51.5%)	3.40 (2.37–4.88)	0.000*
Greater than 500 liters	1934	460 (23.8%)		
Lactating cows (%)				
Less than 30	37	9 (24.3%)	-	0.001*
From 30 to 60	390	133 (34.1%)	1.61 (0.71–3.99)	
Greater than 60	1637	385 (23.5%)	0.59 (0.47–0.76)	
Cows up to the 3rd lactation (%)				
0 to 59	427	142 (33.3%)	-	0.000*
From 60 to 79	405	187 (46.2%)	1.72 (1.29–2.30)	
From 80 to 100	1232	198 (16.1%)	0.22 (0.17–0.29)	
Type of milking				
Manual	102	61 (59.8%)	-	0.000*
Mechanical bucket milking	28	6 (21.4%)	0.18 (0.06–0.52)	
Mechanical canalized	1934	460 (23.8%)	1.14 (0.45–3.47)	
Milking location				
Corral	102	61 (59.8%)	4.77 (3.17–7.22)	0.000*
Milk room	1962	466 (23.8%)		
Cleaning of the facilities				
Yes	2025	502 (24.8%)	0.18 (0.09 – 0.35)	0.000*
No	39	25 (64.5%)		

[a] Odds ratio, [b] Confidence interval. * Association significant at 5%.

RESULTS

From the milk samples analyzed, 53 (2.6%) were associated with clinical mastitis and 584 (28.2%) showed reactions of one, two or three scores on the CMT. In the microbiological exam, 527 (25.5%) samples were positive and 662 (32.1%) showed contamination. In 875 (42.4%), any agent was identified. The microorganisms isolated from clinical and subclinical cases of mastitis are shown in Table 1.

The lowest percentage of susceptibility of *Staphylococcus* spp. to the antimicrobial drugs was for amoxicillin (32.6%), followed by ampicillin (33%), penicillin (34%), tetracycline (82.6%), streptomycin (88.1%), doxycycline (88.6%), trimethoprim-sulfamethoxazole (97.8%), erythromycin (98.2%), lincomycin (98.2%), oxacillin (98.2%), ciprofloxacin (99.1%), cephalexin (99.5%), enrofloxacin (99.5%) and gentamicin (99.5%). All the isolates were susceptible to rifampicin and 61 (28%) showed susceptibility to all the drugs tested. The multidrug resistance profile of the *Staphylococcus* spp. according to the properties studied may be observed in Table 2.

In Tables 3 and 4 the results are made available of univariate analysis of the factors of interest associated with the microbiological examination. In logistic regression, the following were identified as risk factors: animal husbandry system (OR=8.13; p=0.000), feed supplementation (OR=2.92; p=0.000), teat drying process (OR=8.11; p=0.000), antisepsis of the teats before (OR=1.93; p=0.005) and after (OR=3.09; p=0.000) milking, and hygiene habits of the milking workers (OR=5.3; p=0.000) (Table 5).

DISCUSSION

The frequencies of clinical and subclinical mastitis are highly esteemed parameters in evaluation of the health of the bovine mammary gland (Fonseca & Santos 2001). In this study, the rate (2.6%) of mammary quarters that showed signs of inflammation or alterations in the milk were greater than that found by Freitas et al. (2005) in dairy cows in the Agreste of Pernambuco (1%) and less than that observed for cows in Mato Grosso (5.8%) (Martins et al. 2010). The greater prevalence of subclinical infection in relation to clinical infection was also verified in other herds of different states (Bueno et al. 2002, Oliveira et al. 2009).

Of the mammary quarters with clinical mastitis and among the reagents in the CMT, 41.5% and 42% were positive in the microbiological culture respectively. According to the literature, this examination may be negative in approximately 15 to 40% of the samples with clinical alterations and is associated with factors such as low concentration or low elimination of pathogens in the milk, intracellular localization of certain agents, spontaneous elimination of the infection and, in some cases, non-infectious mastitis (Olde Reikerink et al. 2008). Furthermore, 260 (12.6%) samples analyzed were negative in the CMT but positive in the culture. This result reinforces the fact that even when triage tests are used on the dairy farms, there are animals

Table 4. Univariate analysis of factors associated or not associated with bovine mastitis, according to hygiene-health management on rural properties Bahia and Pernambuco

Variable	N	Microbiological (Positive)	Univariate analysis OR[a] (IC[b] 95%)	p value
Feeding during milking				
Yes	365	101 (27.7%)	1.14 (0.87 – 1.47)	0.166*
No	1699	426 (25.1%)		
Milking line				
Yes	1943	463 (23.8%)	0.27 (0.19 – 0.40)	0.000*
No	121	64 (52.9%)		
California mastitis test				
Yes	373	109 (29.2%)	1.25 (0.97 – 1.62)	0.042*
No	1691	418 (24.7%)		
Strip cup test				
Yes	2010	494 (24.6%)	0.20 (0.11 – 0.37)	0.000*
No	54	33 (61.1%)		
Calf present				
Yes	498	245 (49.2%)	4.40 (3.54 – 5.48)	0.000*
No	1566	282 (18.0%)		
Washing of teats before milking				
Yes	1606	302 (18.8%)	0.23 (0.19 – 0.29)	0.000*
No	458	225 (49.1%)		
Drying of teats				
Yes	2011	489 (24.3%)	0.12 (0.06 – 0.23)	0.000*
No	53	38 (71.7%)		
Drying process				
Paper towel	1971	469 (23.8%)	-	0.000*
Cloth towel	40	20 (50.0%)	3.20 (1.62 – 6.33)	
Not used	53	38 (71.7%)	2.53 (0.98 – 6.56)	
Antisepsis of the teats before milking				
Yes	1971	469 (23.8%)	0.18 (0.12 – 0.28)	0.000*
No	93	58 (62.4%)		
Antisepsis of the teats after milking				
Yes	1566	282 (18.0%)	0.22 (0.18 – 0.28)	0.000*
No	498	245 (49.2%)		
Disinfectant rotation				
Yes	730	268 (36.7%)	2.40 (1.96 – 2.94)	0.000*
No	1334	259 (19.4%)		
Antimicrobial agent rotation				
Yes	334	84 (25.1%)	0.97 (0.73 – 1.28)	0.460
No	1730	443 (25.6%)		
Hygiene habits of the milking workers				
Yes	1971	469 (25.1%)	0.18 (0.12 – 0.28)	0.000*
No	93	58 (62.4%)		
Training of milking workers				
Yes	2010	494 (24.6%)	0.20 (0.11 – 0.36)	0.000*
No	54	33 (61.1%)		

[a] Odds ratio, [b] Confidence interval. * Association significant at 5%.

Table 5. Multivariate analysis of the risk factors associated with bovine mastitis on rural properties in Bahia and Pernambuco

Variables	p value	OR[a]	IC[b] 95%		Coefficient	SE[c]
Feed supplement						
No/Yes	0.000*	2.92	1.59	5.60	1.096	0.320
Animal husbandry system						
Extensive/Intensive	0.000*	8.13	4.17	15.83	2.095	0.340
Semi-intensive/Intensive	0.000*	4.19	3.35	5.24	1.433	0.114
Teat drying process						
Cloth towel/Paper towel	0.000*	3.20	1.70	6.00	1.164	0.320
Not used/Paper towel	0.000*	8.11	4.42	14.87	2.093	0.309
Antisepsis of the teats before milking						
No/Yes	0.005*	1.93	1.21	3.06	0.658	0.236
Antisepsis of the teats after milking						
No/Yes	0.000*	3.90	3.09	4.93	1.362	0.119
Hygiene habits of the milking workers						
No/Yes	0.000*	5.30	3.44	8.17	1.669	0.220

[a] Odds ratio, [b] Confidence interval, [c] Standard error of the estimate. * Association significant at 5%.

that may harbor mastitis causing agents (Kapronezai et al. 2005).

The microorganisms identified in this study were similar to those reported by other researchers in different regions of Brazil (Barbalho & Mota 2001, Ferreira et al. 2007, Langoni et al. 2011). *Staphylococcus* spp. and *Corynebacterium* spp. were the main agents diagnosed on all the properties visited, where management faults were verified, such as inadequate hygiene practices of the hands of the milking workers (farms D, E, F) and of the milking equipment (B, G, H), as well as lack of performing antisepsis of the teats after milking (B, D, E, F). These procedures are closely associated with transmission of contagious mastitis during milking (Fonseca & Santos 2001) and may explain the significant number of these pathogens in the samples studied.

In spite of reports regarding the isolation of *Prototheca* spp. in cases of mastitis in Brazil (Mota et al. 1999, Amorim et al. 2010), it is not a very common finding. In this study, this alga appeared as the main agent recovered from clinical infections, corresponding to 45.4% of the cases in which there was microbial growth. All the isolates of *Prototheca* spp. came from the H property, where a large number of cows were milked mechanically and the grazing areas were excessively dirty with organic matter. As the collection of samples was performed in a rainy period, this suggests that the infections occurred due to broad dissemination of algae in the environment of the animals (Bueno et al. 2006). Furthermore, the presence of Gram negative bacteria in the samples evaluated indicates the opportunistic behavior of these microorganisms in the establishment of mastitis, among which *E. coli* and *K. pneumoniae* have been most observed in clinical and subclinical cases of the disease (Langoni et al. 2011).

Low rates of *in vitro* susceptibility to amoxicillin, ampicillin and penicillin were also found in *Staphylococcus* spp. from bovine mastitis in Pernambuco (Freitas et al. 2005) and São Paulo (Nader Filho et al. 2007). In most of the properties studied, the beta-lactams were the drugs of choice for therapy of intramammary infections, such that frequent and often inadequate use of these medications has probably contributed to selection of resistant bacteria in the herds. On the other hand, the microorganisms showed susceptibility percentages above 80% for the rest of the drugs evaluated, especially for cephalexin, gentamicin and enrofloxacin. Other authors (Nader Filho et al. 2007, Medeiros et al. 2009) described similar findings, suggesting that these antimicrobial agents could provide good *in vivo* effectiveness in the treatment of staphylococcus mastitis. Furthermore, susceptibility to all the active ingredients tested was seen in 28% of the isolates of this study; this situation was described in 37.8% of the *Staphylococcus* spp. of mastitis milk analyzed by Medeiros et al. (2009).

Resistance to three or more drugs was observed in 65.6% of the *Staphylococcus* spp., differing from Nader Filho et al. (2007) and Ribeiro et al. (2009), who reported percentages of 48.6% and 39.6% for the bacteria analyzed, respectively. In spite of the small number of isolates coming from properties A (n=5) and D (n=9), all of them

showed multidrug resistance; this was observed in 90.5% and 71.7% in farms G and B, respectively. In all the herds, most (80.4%) of the bacteria were resistant to three or four antimicrobial agents simultaneously, principally from the beta-lactam class. Multidrug resistance to five, six or seven drugs was found in 28 (19.6%) *Staphylococcus* spp., 67.9% of which came from farm B. Such isolates also showed a characteristic resistance profile for the tetracyclines and streptomycin, which was little pronounced on the other properties and is associated with the use of these medications for treatment of infectious diseases in the animals of the herd in question.

Some production characteristics were identified as risk factors for the occurrence of mastitis on the properties. A significantly greater frequency of positive samples was seen on the microbiological examination for animals maintained in an extensive production system (OR=8.13; $p<0.05$) or semi-intensive production system (OR=4.19; $p<0.05$). Some authors affirmed that cows raised intensively are more susceptible to the development of intramammary infections through the greater concentration of animals and exposure to organic matter and to pathogenic microorganisms (Kalmus et al. 2006). In spite of that, we believe that the results of this study are associated with deficiencies in nutritional and hygiene-health management of the animals and of the facilities, with little adoption of measures for control and prevention of mastitis in the herds analyzed. In addition, the lack of feed supplements (OR=2.92; $p<0.05$) was also indicated as a risk factor for the disease, considering that insufficient ingestion of certain vitamins and minerals may negatively affected the immunological resistance of the cows through causing alterations in the mechanisms related to the leukocyte function and to the integrity of the mammary tissue (Heinrichs et al. 2009).

Among the variables associated with milking management, the teat drying process, the lack of performing antisepsis of the teats and inadequate hygiene habits of the milking workers constituted risk factors for mastitis (Table 5). The use of cloth towels for drying the teats after washing is not recommended due to the possibility of transmission of microorganisms to the udder; such microorganisms may be disseminated among the animals, especially if they are used for multiple cows. On the other hand, the practices of disinfection of the teats before and after milking proved to be effective in elimination of surface agents of the mammary gland, contributing to reduction in the incidence of mastitis in the herds. Moreover, adjustment of the hygiene habits of the milking workers, directing them to wash their hands with soap and water before and during milking, is an essential measure for prevention of intramammary infections (Fonseca & Santos 2001).

In spite of not being confirmed as risk factors, other factors that showed significant association ($p<0.2$) are worthy of note. The percentage of positive microbiological examinations was greater for the samples in which milking was performed with the presence of the calf. According to Brito et al. (2000), sucking by the calf promotes an increase in the colonization of microorganisms from the oral cavity in the skin of the teats; nevertheless, Oliveira et al. (2011) affirmed that this procedure may reduce the rates of mastitis due to the removal of residual milk from the mammary gland and the antimicrobial action of the saliva. In addition, these authors observed that the feeding of animals during milking contributed to the increase in the occurrence of infection in cows from Minas Gerais (Souza et al. 2005) and Pernambuco (Oliveira et al. 2012) since soon after the milking, the teat sphincter remains open, favoring the entrance of environmental pathogens.

CONCLUSIONS

On the rural properties studied, there is a predominance of subclinical and clinical infections caused by *Staphylococcus* spp. and *Prototheca* spp. respectively.

The presence of multiresistant isolates in bovine milk shows the importance of adequate choice and use of antimicrobial agents, with a view towards success in the treatment of mastitis.

The risk factors identified are mainly associated with deficiencies in management during milking.

Control and prevention measures, including antisepsis of the teats and good practices for achieving hygienic milking should be established for the purpose of preventing new cases of the disease in the herds.

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