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Original Article

Etiological profile of early neonatal bacterial sepsis by multiplex qPCR

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Abstract

Introduction: Given the major impact in terms of morbidity and mortality that episodes of early neonatal sepsis (ENS) have on both newborns and health systems, this study aimed to identify the etiological profile of early neonatal bacterial sepsis by a multiplex quantitative real-time polymerase chain reaction (qPCR).

Methodology: Blood samples from newborns diagnosed with clinical ENS and hospitalized in neonatal intensive care units (NICUs) were collected and analyzed using the multiplex qPCR method to detect *Streptococcus agalactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter* sp., *Serratia* sp., and *Staphylococcus aureus*. A universal primer was used in the analysis.

Results: A total of 150 neonates with clinical sepsis and 10 newborns as healthy controls were included in the study. The group with clinical sepsis was 100% positive for the presence of bacterial genomic DNA through the universal primer. The control group showed negativity by qPCR. The multiplex qPCR analysis showed that 76% of the samples were positive for *Escherichia coli*, 34% for *Staphylococcus aureus*, 13.3% for *Streptococcus agalactiae*, 7.3% for *Pseudomonas aeruginosa*, and 0.7% for *Enterobacter* sp. and *Serratia* sp. Multiplex qPCR of patients with clinical sepsis matched with 8.1% of the blood samples that tested positive by the microbiological method.

Conclusions: Rapid and sensitive detection of the pathogens causing ENS by this new multi-target approach based on multiplex qPCR could potentially excel compared to microbiological methods, with the simple objective of facilitating the progression to a more rapid and specific antimicrobial therapy, avoiding the abuse of antibiotics in NICUs.

Key words: early neonatal sepsis; multiplex qPCR; multi-target qPCR; molecular diagnosis.

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Introduction

Sepsis is still a major cause of morbidity and mortality in the neonatal period, particularly in preterm infants [1]. Early signs and symptoms of infection are generally minimal and can easily be misinterpreted as being due to non-infectious causes. However, although the onset of the disease is often unspecific, the clinical course may be fulminant, leading to septic shock, disseminated intravascular coagulation, and death within hours of initial clinical symptoms [2].

When the etiology of sepsis is bacterial, the most common form, confirmatory diagnosis depends on microbiological tests based on blood culture techniques, which, although still considered the gold standard, usually take 48 to 72 hours to yield results and have low sensitivity [3]. Thus, in most cases, antibiotic therapy is initiated empirically, based on clinical criteria and knowledge of the most common local microbiota [4].

A fast and correct diagnosis accompanied by prompt treatment is an important factor in reducing mortality from neonatal sepsis. Empirical therapy leads to the indiscriminate use of antibiotics, which may induce bacterial resistance and increase yeast infection rates, lifting treatment costs and increased mortality, especially in extremely preterm infants [5].

Unfortunately, no clinical signs or laboratory tests currently available can detect early neonatal sepsis [6]. Thus, faster, specific, and sensitive tests are needed to prevent the inappropriate use of antibiotics, streamlining the etiological diagnosis of sepsis and thereby allowing the implementation of safe and appropriate therapeutic management for each case [7].

Strategies for nucleic acid amplification by polymerase chain reaction (PCR) focus on rapid objective methods and the specificity in the diagnosis of infections, which are very difficult to detect. Thus, its efficacy, safety, and speed can turn them into essential tools for a more specific and effective treatment, favoring a reduction in treatment time, morbidity, mortality, and hospital costs. The application of such techniques to detect and identify suspected infection has the potential to revolutionize the diagnosis and management of sepsis [8,9].

Unlike microbiological cultures, PCR diagnosis confirms the presence or absence of bacterial genomic DNA, which may be highly related to clinical sepsis in newborns. Such diagnosis may be available to the clinician in a few hours [5]. However, there have been only a few studies that used molecular tests based on multiplex real-time quantitative PCR in blood samples for diagnosis of neonatal sepsis, and there are no studies to date on ENS.

Given the major impact in terms of morbidity and mortality that episodes of ENS results have for both newborns and for health systems, this study aimed to identify the etiological profile of early neonatal bacterial sepsis. Multiplex real-time PCR was applied, with the objective of contributing to the elaboration of a more rational and specific therapy for this serious condition.

Methodology

Design, location, and study population

This was a cross-sectional observational descriptive study. The population consisted of newborns diagnosed with clinical sepsis admitted to the neonatal intensive care units (NICUs) of the following institutions: University Hospital of the Federal University of Mato Grosso do Sul (HU), Santa Casa de Campo Grande (SCCG), and Regional of Mato Grosso do Sul Hospital (RH), from September 2013 to February 2014.

The study was approved by the Ethics Committee on Human Research of the Federal University of Mato Grosso do Sul (UFMS) under nº 355.636/CAAE 16465613.7.0000.0021 protocol on 09/08/2013. Demographic and clinical and laboratory data of newborns were collected through standardized structured forms, using information obtained through interviews with the mother and analysis of information in the patient's record. Gestational age was estimated according to the method of New Ballard [10].

The study included newborns of any gestational age who were diagnosed with clinical sepsis at birth for the period up to 72 hours of life and who received systemic empirical antibiotic to treat ENS.

The clinical diagnosis of early neonatal sepsis [11] was defined based on the presence of three or more clinical signs, including apnea, difficulty breathing, cyanosis, tachycardia or bradycardia, perfusion deficit or shock, hypo- or hyperglycemia, irritability, lethargy, hypotonia, seizures, abdominal distension, vomiting, intolerance diet, gastric residue, hepatomegaly, jaundice of unknown cause, unstable temperature, petechiae or purple skin, and an unwell appearance, or at least two of these signals associated with maternal risk factors such as fever > 38°C, chorioamnionitis, premature rupture of membranes greater than 18 hours, colonization by group B *Streptococcus* and urinary tract infection. Chorioamnionitis was diagnosed when there was evidence of pathogenic bacteria in amniotic fluid per gram or culture, amniotic purulent fluid, leukocytosis, maternal fever, or pain/uterine discomfort.

A control group was included, which consisted of randomly selected newborns at term who were admitted to the maternity ward of SCCG, from January to February 2014, who did not present any risk factor for early onset sepsis and were found to be asymptomatic. Newborns with congenital malformations and/or maternal infection during pregnancy with human immunodeficiency virus (HIV), syphilis, toxoplasmosis, rubella, cytomegalovirus, and/or herpes virus, were excluded.

Blood samples

All infants enrolled in the study underwent blood sampling for aseptic blood culture and PCR after suspected clinical ENS and before the commencement of systemic empirical antibiotic therapy. Blood samples for PCR, collected in EDTA, were stored in a freezer at -80°C until DNA extraction for subsequent realization of molecular technique. The blood cultures were performed using the automated system BacT Alert (Organon Teknika Corporation, Durham, USA).

For analysis of blood count and levels of C-reactive protein (CRP), 0.3–1 mL of whole blood was collected by peripheral vein or arterial umbilical catheter after 18 hours of life, according to routine procedures of each neonatal unit.

PCR oligonucleotide primers

The oligonucleotide primers selected for qPCR were designed from specific genomic sequences of the GenBank database (National Center for Biotechnology Information) for seven bacteria: *Streptococcus*

agalactiae, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter* sp., *Serratia* sp., and *Staphylococcus aureus*. Also included in the multiplex qPCR was a non-specific universal primer pair, based on identifying regions within the 16S rRNA gene of all bacterial genomic DNA, as described elsewhere [12,13]. All designed primers were analyzed by BLASTn software before being synthesized by IDT (Integrated DNA Technologies, Coralville, USA). Pairs of oligonucleotide primers, as well as respective target sizes, are shown in Table 1.

DNA extraction

For genomic DNA extraction from clinical specimens and isolation from bacteria investigated, Illustra genomic Prep Blood Mini Spin Kit (GE Healthcare, Piscataway, USA) was used according to the manufacturer's recommendations. The extracted DNA was quantified at 260 nm in a GeneQuant spectrophotometer (GE Healthcare, Piscataway, USA).

Multiplex qPCR

To perform multiplex real-time PCR, a SYBR Green PCR Core Reagents Kit (Invitrogen Life Technologies, Carlsbad, USA) was used. The qPCR amplification reactions followed the protocol described by Anbazhagan *et al*. [14] with some modifications. In a final total of 25 µL reaction, 3 pmol of each primer, 10 ng genomic DNA from clinical specimens, 12.5 µL SYBR Green PCR Master Mix (Invitrogen Life Technologies, Carlsbad, USA), and deionized water were included. The reactions were performed in triplicate, under standard thermocycling conditions in the AB ViiA 7 Real-time PCR System (Applied Biosystems, Warrington, UK). The melting curves were generated for each clinical specimen, as well as for positive and negative controls and standards, all in the same reaction plate. A specific Tm for each amplified target was further confirmed.

As a negative control, autoclaved ultrapure water (Milli-Q, Ultrapure, Millipore Synergy with UV, Loveland, USA) was used. As a positive control, purified total DNA of microorganism patterns obtained from microbiological culture collections was used, put up as colonies of each bacterial species in their respective vials with 1 mL of lysing solution and subsequent DNA extraction. All isolates of bacteria surveyed were chosen from isolated cultures in the HR Microbiology Sector during 2014. All pathogens were grown in specific media at 37°C for 24 hours before extraction.

Statistical analysis

The variables related to maternal characteristics and clinical and epidemiological characteristics of newborns are presented in descriptive format. Statistical analysis was performed using Sigma Stat version 3.5 and Systat version 12.0 software programs. To assess whether the numerical data had normal distribution or not, the Kolmogorov-Smirnov test was used. When the distribution was shown to be non-parametric, the Mann-Whitney test was used to compare the clinical sepsis and control groups. A value of $p \leq 0.05$ was considered statistically significant.

Table 1. Multiplex qPCR oligonucleotide primers designed for the diagnosis of early neonatal sepsis (ENS).

Primer	Microorganism	Product size (bp)
ECLOACFOR = 5'-GTC TAT TTC GCA CGT CGT GCT TTG C-3' ECLOACREV = 5'-CTT CTC AAC TGC GCG GAT GAG ACC-3'	*Enterobacter* spp.	171
SAUFOR = 5'-GAG GTA TTA GTG ATA TCG ATT TAA-3' SAUREV = 5'-GCT TTA ATG TCA CTT GTA TTG AT-3'	*Staphylococcus aureus*	139
SERFOR = 5'-CAG CGG CTC GGC GCC GTT GAT-3' SERREV = 5'-TTC GTC GCC GAC GAC GCA CTG-3'	*Serratia* spp.	120
PSEUDFOR = 5'-CCA GCC ATG CCG CGT GTG TGA-3' PSEUDREV = 5'-GTT GGT AAC GTC AAA ACA GCA AGG-3'	*Pseudomonas aeruginosa*	102
ECOFOR = 5'-CAT CAG ATG GAG CCG GGC ATG CCA-3' ECOREV = 5'-GAG AAT TAC TCG TCT TCC AGT TCG-3'	*Escherichia coli*	98
SAGAFOR = 5'-TTG CAG CCA GTT GAA GAT CGT TAT-3' SAGAREV = 5'-TAA TTC CAT AAA TCG CTT TGT ATC-3'	*Streptococcus agalactiae*	90
KLEBFOR = 5'-GCA CTG CGT GGT GAT GTC GC-3' KLEBREV = 5'-TGT AAC GAC GGG CAA TCT TCA-3'	*Klebsiella pneumoniae*	82
RW01 = 5'-AAC TGG AGG AAG GTG GGGAT-3' DG74 = 5'-AGG AGG TGA TCC AAC CGCA-3'	Universal	380
PSEUDFOR2 = 5'-GCC GCG TGT GTG AAG AAG GTC TTC-3' PSEUDREV = 5'-GTT GGT AAC GTC AAA ACA GCA AGG-3'	*Pseudomonas aeruginosa*	100
SAUFOR = 5'-GAG GTA TTA GTG ATA TCG ATT TAA-3' SAUREV2 = 5'-ATG CCT GCT TTC AAA TCG ACA ACT C-3'	*Staphylococcus aureus*	139

Table 2. Characteristics related to newborns in the control group and in the early neonatal clinical sepsis.

Variable	Control n (%)	Sepsis n (%)
Antenatal *S. agalactiae* screening	0 (0)	3 (2)
Antepartum use of antibiotic	0 (0)	100 (66.7)
UTI in the 3rd trimester[b]	0 (0)	93 (62)
MR ≥ 18 hours before delivery[c]	0 (0)	37 (24.7)
Preterm labor	0 (0)	76 (50.7)
Chorioamnionitis	0 (0)	9 (6)
Rupreme	0 (0)	51 (34)
Cesarean	02 (20)	94 (62.7)
Male	5 (50.0)	81 (54.0)
Gestacional age < 37 weeks	10 (100)	39 (26.0)
Birth weight		
≥ 2,500 g	10 (100)	47 (31.3)
1,500–2,499 g	0 (0)	56 (37.4)
< 1,500 g	0 (0)	47 (31.3)
Clinical signs		
Respiratory distress	0 (0)	145 (96.6)
Apnea	0 (0)	50 (33.3)
Poor perfusion	0 (0)	35 (23.3)
Apgar in the 5th minute < 7	0 (0)	6 (4.0)
Total	10 (100)	150 (100)

UTI: urinary tract infection; MR: membrane rupture.

Results

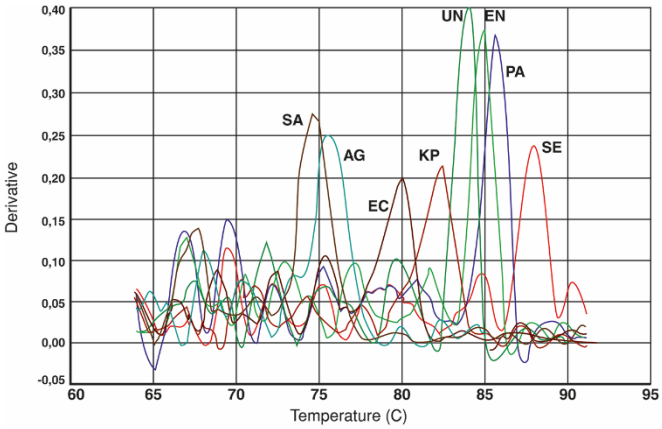
A total of 174 newborns of both genders diagnosed with clinical sepsis were evaluated. Of these, 24 newborns who met the exclusion criteria (2 where it was not possible to obtain informed consent, 8 who had some congenital malformation, 4 in which the mother had syphilis during pregnancy history, and 10 newborns who had not received the full course of antibiotics) were excluded.

Table 2 describes maternal characteristics, care, clinical and epidemiological characteristics related to newborns with clinical sepsis and the control group included in this study. Table 3 describes the laboratory findings among the included infants.

DNA samples of patients and controls were initially screened for the presence of bacterial microorganisms through the universal primer, with positivity demonstrated in the group of 150 samples with clinical ENS and negativity in the 10 samples of the control group.

DNA samples from patients were tested for the presence of DNA of bacterial microorganisms selected through specific primers, using multiplex qPCR (heptaplex); the target bacteria were found in 97% of the samples. The amplification of specific fragments using standard dissociation curves can be seen in Figure 1. In contrast, the blood culture was negative in 97% of

Figure 1. Typical results obtained by multiplex qPCR. The graph shows the specific dissociation curves.



SA: *S. aureus*; AG: *S. agalactiae*; EC: *E. coli*; PA: *P. aeruginosa*; KP: *K. pneumoniae*; EN: *Enterobacter* sp.; SE: *Serratia* sp.; UN: universal primer

Table 3. Laboratory findings related to newborns in the control group and in the early neonatal clinical sepsis group.

Laboratory findings	Control [a]	Sepsis [a]	p [b]
Leukocytes	16,150 (3,930)	12,325 (8,050)	0.056
Neutrophils	10,508 (4,015)	7,381 (2,376)	0.064
Platelets	212,500 (104,000)	215,000 (107,000)	0.514
CRP (mg/dL)	0.8 (0.4)	4.7 (12,6)	0.133
I/T ratio [d]	0.005 (0.02)	0.04 (0.07)	0.053

[a] Results presented by median ± half-width. [b] Statistical analysis using the Mann-Whitney test; CRP: C-reactive protein; I/T: immatures/total neutrophils.

cases for *S. agalactiae* (2 cases), the bacteria most commonly found between positive blood cultures, followed by *P. aeruginosa* and *Serratia* sp., with a case for each.

After the analysis of dissociation and amplification curves, it was possible to infer that 114 samples were positive for *E. coli* (76%), 52 (34.7%) for *S. aureus*, 20 samples (13.3%) for *S. agalactiae*, and 11 samples were positive for *P. aeruginosa* (7.3%). *K. pneumoniae* was observed in 8 samples, and *Enterobacter* sp. and *Serratia* sp. were each found in 1 sample. Figure 2 shows the final results of the multiplex qPCR in the selected population.

Of the 150 samples analyzed, 72 (48%) were positive for only one bacterium. The remainder of the samples was positive for more than one bacteria, among which the most commonly found was the association of *S. aureus* and *E. coli*, in 28.6% of cases.

The multiplex qPCR patients with clinical sepsis were confirmed microbiologically by 3% in the blood samples. The concordance rate between the multiplex qPCR and positive blood cultures for the same bacteria was 75%. A patient with positive blood cultures for *P. aeruginosa* was not positivie by multiplex qPCR for the same bacteria.

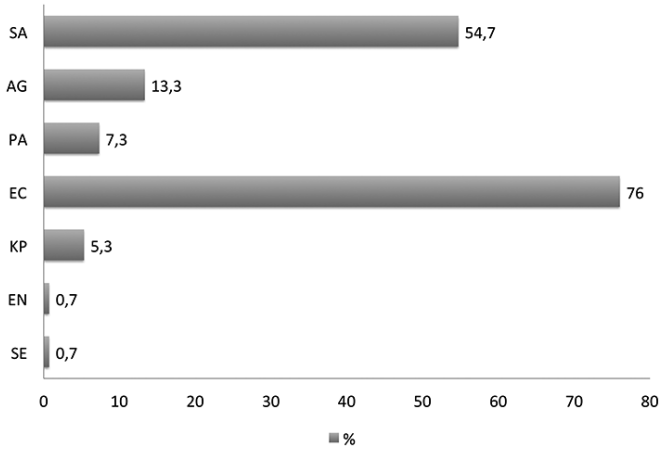
Discussion

The increase in neonatal mortality presents a major challenge to global public health [15]. Neither treatment nor results with respect to the neurodevelopment of surviving newborns with sepsis have changed significantly over the last 30 years [16]. Thus, new diagnostic techniques, among which the molecular method shows up faster and is more specific and sensitive than blood culture in the diagnosis of neonatal sepsis [17,18], have the potential to replace the current microbiological methods.

Given the extent of the problem and the major impact in terms of morbidity and mortality that episodes of sepsis cause in newborns, this study presents a bacterial etiological profile through multiplex qPCR of the blood samples of newborns with clinical diagnosis ENS in order to provide insight into more agile and effective identification and treament through a more specific and early diagnosis of these patients.

A complete blood count with global and specific leukocyte count is commonly performed in the evaluation of ENS in the NICU. However, many factors can affect these counts [16]. Hornik *et al*. [19] retrospectively examined the usefulness of hematological indices in more than 200,000 newborns and concluded that such rates did not show sufficient utility to identify patients with ENS. In this study, differences were not observed in the number of leukocytes, neutrophils, platelets, CRP, and immature neutrophil ratio on the total between the group with clinical sepsis and the control group, which confirms the low importance that these parameter features in the diagnosis of ENS.

Figure 2. Prevalence of target microorganisms detected by multiplex qPCR.



SA: *S. aureus*; AG: *S. agalactiae*; EC: *E. coli*; PA: *P. aeruginosa*; KP: *K. pneumoniae*; EN: *Enterobacter* sp.; SE: *Serratia* sp.

Microbiological blood culture is still considered the gold standard in the diagnosis of neonatal sepsis, but its sensitivity is usually low in newborns, regardless of its delay of 48–72 hours in showing positive results [17].

In this study, the blood culture positivity rate was 3%, similar to that found by Al-Taiar *et al*. [20], who found a positive rate of 4.6%. Other studies, however, described higher rates of positivity in their blood cultures, such as the 23.59% found by Patel *et al*. [21] and 42% observed by Hussein and Khaled [22]. The low positivity rate can be influenced by several factors, such as the technique used in the analysis of cultures in each service, the existing limitation on the blood volume obtained for culture due to weight and clinical condition of the patient (which was most often premature), and the observed high rate of use of antenatal antibiotics [22].

The qPCR in this study showed positivity in all patients (100%), when using universal primer, noting that in 97% of patients studied, we detected genomic DNA of one or more from among the seven bacteria investigated. It should be noted, however, that a positive result for the presence of genomic DNA in the blood does not necessarily mean that the patient is presenting sepsis by that bacteria and can only suggest a possible bacterial colonization. However, the true

sepsis risk increases considerably, when bacteria DNA is detected in a patient showing risk factors and clinical signs of infection. Furthermore, the risk of sample contamination should be considered in the analysis of such results; this risk was minimized by the use of aseptic technique during sample collection and during all stages of the qPCR.

The concordance of positive blood cultures to detect the bacteria in the same multiplex qPCR in patients with clinical sepsis was 75%, a result similar to that found by Bloos *et al*. [24], who described a concordance of 70%. On the other hand, the correlation of heptaplex qPCR positive results confirmed by blood culture was only 3%. In this case, the latter authors found greater concordance of 21%. This suggests that the presence of bacterial DNA does not always have pathophysiological significance and does not necessarily indicate the presence of viable bacteria in the blood. Based on the known limitations of blood culture, the question of the significance of the presence of genomic DNA in the blood of patients with clinical sepsis becomes of great importance, requiring molecular studies that better evidence the expression of the living presence of the detected microorganism.

The findings of the present study suggest that *E. coli*, *S. aureus*, and *S. agalactiae* are the main causative agents of ENS. There was a predominance of Gram-negative bacteria in the samples. This prevalence, as well as the finding that *S. aureus* was the most commonly found Gram-positive bacteria, confirms the findings described by Zaidi *et al*. [25] in their study of neonates with ENS in Latin America, the Caribbean, Asia, and Africa. In Brazil, the study by Paes *et al*. [26] and Silva *et al*. [27], both held in Pará State, also demonstrated a prevalence of Gram-negative bacteria among patients with ENS, which was also observed by Fahmey [28] in India; however, in the latter study, *K. pneumoniae* was the predominant bacteria found. The study by Sharma *et al*. [29] also demonstrated that *S. aureus* and *E. coli* were among the main bacteria found in ENS. It is suggested that after the introduction of antepartum prophylactic antibiotics, Gram-negative bacteria, especially *E. coli*, have become both more common, especially among the preterm population [30].

On the other hand, one could not exclude early onset of fungal sepsis. Though found infrequently in early neonatal sepsis, even in the NICU setting, fungal infections, most commonly involving *Candida* sp., are more frequently associated with late-onset sepsis. The incidence of *Candida* species ENS in NICUs has been reported to be 1.4% [31]. In the present study, fungi were not included, but they are indeed a very important molecular target. Nevertheless, according to the local microbiological and epidemiological data, fungal species may be definitely included in some multiplex qPCR testing to increase diagnostic capabilities and cover all the most common causes of ENS. For instance, previous studies of multiplex qPCR indicated an excellent capacity for detecting antifungal microorganisms whenever necessary [32-33].

It is noteworthy that this is the first study to describe the etiological profile of a population of neonates with ENS through the molecular method of qPCR, for which a comparative analysis was done with studies using blood culture rather than molecular methods.

However, it is believed that the development of this new multi-target approach, based on multiplex qPCR for the rapid and specific detection of important pathogens causing ENS, can stand out as a potentially useful tool in clinical emergencies in comparison with microbiological culture-based methods, with the objective of facilitating progression to antimicrobial therapy that is specific, which is especially relevant in newborns, as empirical treatment is often abused in neonatal intensive care units.

Conclusions

The method of multiplex qPCR showed great capacity to detect the DNA of the seven bacteria surveyed in suspected ENS, and all samples were positive in the multiplex qPCR using the universal primer. Blood culture results showed very low sensitivity, discordant with clinical manifestations in patients, thus strengthening the molecular method as the choice in the identification and treatment of suspected diagnosis of early sepsis.

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