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The Use of Matrix-assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry for the Identification of the Bacterial Agents Involved in Subclinical Endometritis in Female Dromedary

Derar Derar[1,2], Ahmed Ali[1,2], Elhassan M.A. Saeed[1], Fahd Al-Sobayil[1], Ayman Elbehiry[3,4] 10.18805/IJAR.BF-1542

ABSTRACT

Background: This study aimed to identify different bacterial isolates incriminated in subclinical endometritis (SCE) in female dromedary using Matrix-Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS) and to evaluate the efficacy of two protocols to treat this condition.

Methods: Subclinical endometritis was detected in 211 dromedaries using cytological examinations. Two hundred twenty-six microbial isolates were obtained from 185 samples. SCE-females were arbitrarily allocated into two groups; group I received intrauterine infusion of 500 mg cephapirin benzathine (MTC, n=42) and group II infused with intrauterine flushing of povidone iodine 10% (PVP-I, n=67).

Result: The MALDI-TOF MS was able to identify 224/226 (99.1%) bacterial isolates to the genus level and 181/226 (80.1%) were identified to the species level. The most common identified bacterial species were *Staphylococcus* aureus (32.74%), *Corynebacterium* sp. (19.03%) and *Escherichia coli* (18.58). Conception rate did not differ between MTC- and PVP-I-treated groups. However, the first service-conception was higher, numbers of services per conception were fewer and treatment to conception interval was shorter in the MTC than PVP-I group. Based on the present data, it can be concluded that MALDI-TOF is efficient, fast and reliable for the detection and identification of various bacterial agents incriminated in subclinical endometritis in female dromedary. Fertility indices in treated female dromedaries associated with subclinical endometritis favors the use of intrauterine infusion of cephapirin benzathine therapy in the present study.

Key words: Cephapirin benzathine, Female dromedary, MALDI-TOF, Povidone-iodine, Subclinical endometritis.

INTRODUCTION

Subclinical endometritis is not uncommon in camels. It is characterized by more or less long-lasting infertility with repeat breeding and low conception rate (Derar *et al*., 2017, 2020). Cytological examination is the most trusted test for the diagnosis of SCE in different animal species such as horses (Overbeck *et al*., 2011) and cattle (Ricci *et al*., 2017).

It was recommended that combined cytological and bacteriological examinations are better than individual tests for the diagnosis of SCE in female camel (Derar *et al*., 2020). As true methods for diagnosing various types of microorganisms, biochemical and genetic analyses are still used, however, their use may be time consuming and expensive (Elbehiry *et al*., 2016). Therefore, it is vital to develop low-cost, fast and accurate methods for detecting different microbes responsible for infectious or non-infectious diseases. In other words, the ability to accurately and rapidly differentiate microorganisms in medical and veterinary diagnostics is a critical step toward developing suitable methods for dealing with contagious infections (Hays *et al*., 2012; Elbehiry *et al*., 2016).

In the United States, MALDI-TOF MS is not only the most widely applied technique for detection of microorganisms, but it is also the most widely used technique for identifying microorganisms (El-Bouri *et al*., 2012). Detection of microorganisms by such technique can be useful for different types of microorganisms (Barreiro *et al*., 2010). Basically, this technology involves shooting lasers at microbes to ionize their proteins, which creates a spectrum of peaks. The associated software of Microflex LT scans the database for a match with microbial species on the basis of a consistent list between the two spectra within the spectra database stored in its library (Pavlovic *et al*., 2013).

[1]Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Qassim, Kingdom of Saudi Arabia (KSA).
[2]Department of Theriogenology, Faculty of Veterinary Medicine, Assiut University, Egypt.
[3]Department of Public Health, Microbiology, College of Public Health, Qassim University, Qassim, Kingdom of Saudi Arabia (KSA).
[4]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Egypt.

Corresponding Author: Derar Derar, Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Qassim, Kingdom of Saudi Arabia (KSA).
Email: dr.mohammad@qu.edu.sa

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The main objectives of the present study were to investigate the credibility of MALDI-TOF-MS for the identification of different microbial agents involved in subclinical endometritis in female dromedary and to evaluate the efficacy of two treatment protocols used to resolve this disorder.

MATERIALS AND METHODS

This study was carried out at the university teaching hospital, Qassim University, Qassim, Saudi Arabia during the breeding season November-April 2020.

Criteria used to diagnose SCE

Subclinical endometritis was diagnosed in 211 barren female dromedaries based on pre-proposed findings. These findings include history of regular repeat breeding for at least three consecutive times; clinically normal genital tract; ≥5% polymorphnuclear cells on cytological examination (Derar *et al*., 2017).

Endometrial swabbing and bacteriological examination

A long double sleeved hand was inserted intravaginal to sample the uteri of the affected animals using sterile swabs (Minitüb, Tiefenbach, Germany). Blood agar was used for primary culture of the bacteriological samples. Each sample was further processed to differentiate between aerobic and anaerobic, fastidious and non-fastidious bacteria and gram-positive and gram-negative, as previously described (Derar *et al*., 2020).

Characterization and clustering of bacteria by MALDI-TOF MS

Sample preparation

MALDI biotyper device (Bruker Daltonik, Bremen, Germany) was used for detection of bacterial species isolated (226 isolates) from the SCE-suspected she-camels. On two spots of target plate, fresh colonies of overnight culture of each isolate were transferred by toothpick and smeared, then each colony was covered with 1 µl of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and air dried at room temperature. Then, MALDI Biotyper Compass software (Bruker Daltonik) produces the mass spectra of the bacteria.

Preparation of the positive control or the bacterial test standard (BTS)

According to the method previously described by Barreiro *et al*. (2010), 50 microliters of the standard solvent was pipetted into the BTS (*Escherichia coli*) pellet of the *in vitro* diagnostic product (IVD) and melted up and down a few times at 25°C, melting the pellet after each pipetting cycle. A five-minute liquefaction of the IVD-BTS solution was then performed 20 times for five minutes and then centrifuged for two minutes at 13,000 RPM. To explore further, 5 µl of the supernatant was transferred to microtubes and stored at -18°C for further testing.

Analysis of the data and clustering

A score value between zero and three is assigned to unidentified spectrum by comparing it with the known spectrum stored in Bruker's library. The strain recognition accuracy was detected on the basis of the measurements taken by Bruker Daltonik. The Microflex LT device successfully detected species when the log score was between 2.3 and 3.00; however, species and genus level were identified in the range of 2.00 to 2.29 and 1.700 to 1.999, respectively. Additionally, a score between 0.00 and 1.69 indicates a lack of trustworthiness in the proof of identity. Spectra were generated using Microflex LT Compass software in a range of m/z values from 2,000 Dalton to 20,000 Dalton. There were 50 laser shots per spot in the official standards. The minimal spanning tree (MSP) data set for the Microflex LT library, which has 5989 bacterial and fungal species, resulted in a dendrogram. Based on the main spectra of different species examined, an MSP dendrogram was generated. A matrix of cross-wise identification scores was derived by comparing the main MALDI spectra with the spectra from the MALDI biotyper taxonomy. The distance values between each pair of main spectra were calculated using this matrix. The Microflex LT Compass software was used to generate a dendrogram based on these values.

Allocation of animals to treatment protocols

According to the treatment protocols, affected animals were randomly assigned into 2 groups. Group I (GI, MTC n=42) received intrauterine infusion of 500 mg cephapirin benzathine according to the manufacturer's instructions (Metricure; Intervet, Whitby, Ont., Canada). Group II (GII, PVP-I, n=67) treated with 1200 ml intrauterine flushing of 10% povidone-iodine (Betadine, Mundipharma, AG-Basel, Switzerland). A 500 mg dose of cloprostenol ($PGF_2\alpha$ analogue, Estrumate, Schering-Plough, Morris Ave, Summit, NJ) was administered intramuscularly to both groups at infusion time. The efficacy of treatment regimens was evaluated for the following fertility indices: i) overall conception rate, ii) conception after the first, iii) no. of services/conception and iv) treatment to conception interval.

Statistical analysis

Data were presented in numbers, means±S.E. or percentages. The SPSS-program, version 25.0 (SPSS Inc., Chicago, IL, USA, 2017) was used for the analysis. Fertility indices were compared with chi-square. Significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Identification of isolates by MALDI-TOF MS

Two hundred and twenty-six microbial isolates were obtained from 185 samples. 26 uterine samples showed no microbial growth. According to the results in Table 1, A total of 176 gram-positive bacteria (n=176) and 50 gram-negative bacteria (n=50) were isolated. Two isolates of gram-negative bacteria were not identified by MALDI-TOF MS although 224/226 (99.1%) bacterial isolates were identified to the species level while 181/226 (81%) isolates were identified to the genus level. The following bacteria were isolated most frequently: *Staphylococcus aureus* (32.74%), *E. coli* (18.58%), *Corynebacterium* sp. (19.03%), *Bacillus pumilus* (10.62%) and *Acinetobacter junii* (10.62%).

In the line spectra, there was evidence of between 10 and 20 noticeable ions peaks located between 2,000 to 16,000 Dalton, with a higher strength peak detected between 3,000 and 10,000 Dalton (Fig 1) that matched various kinds of bacterial strains in the Compass library.

MALDI-TOF MS was used to identify 224 isolates. 97 isolates (43.3%) were identified depending on their score (log) value ranging from 2.3 to 3.0, which was considered a positive identification of their genus and species (Table 2). There were 122 (54.46%) genus or species identifications with a score value of 2.0 to 2.29, whereas only 5 (2.23%) were identified by a score value ranging from 1.7 to 1.9, which was considered probable genus identification.

The gel view (Fig 2) illustrates the spectra of 176 species of gram-positive bacteria (Fig 2A) and 50 species of gram-negative bacteria (Fig 2B). The most peaks were found

Table 1: Identification of various types of bacteria isolated from subclinical endometritis-affected female dromedaries (n=211) by MALDI-TOF MS.

Gram	Isolate	No. of isolates	Prevalence %	Correctly identified
Positive	*Staphylococcus aureus*	74	32.74	74
	Corynebacterium sp.	43	19.03	43
	Acinetobacter junii	24	10.62	24
	Bacillus pumilus	24	10.62	24
	Bacillus subtilis	11	4.87	11
Negative	*Escherichia coli*	42	18.58	42
	Klebsiella pneumoniae	6	2.65	6
	Not identified	2	0.88	0
Total		226	100	224

Table 2: Bacterial species isolated from 211 subclinical endometritis-affected female dromedaries with respective MALDI-TOF MS score.

Category	Score range	Gram-positive						Gram-negative		
		A	B	C	D	E	Total	F	G	Total
1	2.3-3	30	13	12	15	6	76	15	6	21
2	2-2.29	44	30	12	9	5	100	22	0	22
3	1.7-1.9	0	0	0	0	0	0	5	0	5
4	0-1.6	0	0	0	0	0	0	0	0	0

A- *Staphylococcus aureus*; B- *Corynebacterium* sp.; C- *Acinetobacter junii;* D- *Bacillus pumilus*; E- *Bacillus subtilis*; F- *Escherichia coli*; G- *Klebsiella pneumoniae.*

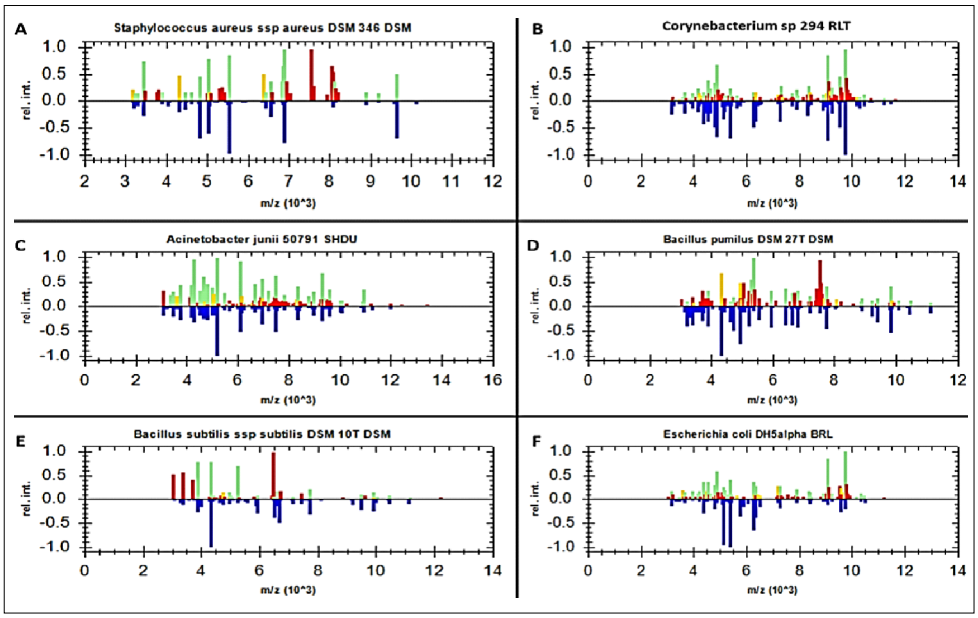

Fig 1: Comparison of mass spectrum protein profiles of unknown samples (green) with 5 reference strains of gram-positive bacteria (*Staphylococcus aureus* (A), *Corynebacterium* sp. (B), *Acinetobacter junii* (C), *Bacillus pumilus* (D), *Bacillus subtilis* (E) and *Escherichia coli* (F) present in database (blue) by Compass software.

between 3,000 and 10,000 dalton and many spectra were scattered between 2,000 and 15,000 dalton. There were some peaks detected between 3,000 and 15,000 Dalton in some gram-positive bacterial species. Gram-negative species, by contrast, showed very weak signals at 15,000 Dalton. Spectra of 224 well-identified gram-positive and negative bacteria were examined by gel view in order to ascertain whether Microflex LT Compass software could distinguish these strains based on their species status. Following this step, a cross-wise minimal spanning tree (MST) dataset was generated out of the various spectra (Fig 3).

Fertility indices

Conception rate did not differ between MTC- and PVP-I-treated groups. On the other hand, the first conception was higher, the number of conceptions was lower and the interval between conceptions and treatments was shorter in the MTC group than in PVP-I (Table 3).

The MALDI-TOF MS technique was already assessed by matching its results with standard conservative procedures and found accurate and reliable (Nagy *et al*., 2012; Osa *et al*., 2021). In the present study, usage of MALDI-TOF MS for the identification of bacteria associated with subclinical endometritis in camels was found successful. The technique was able to identify 99.1% of bacterial isolates to the genus level and 80.1% to the species level, while only two isolates (0.88%) were not identified. Failure to identify these two isolates could be attributed to lack of references in available databases of the technique. Identification scores of all bacterial genera and species were within the manufacturer's recommendations (≥2.0), except five isolates which were identified to the species level as *E. coli*, although their score range was between 1.7 and 2.0. Similar finding of identification to the species level at score of 1.7-2.0 was reported before (Nagy *et al*., 2012).

Table 3: Fertility indices after treatment of SCE-treated female dromedaries with Metricure (MTC n=45) and povidone iodine PVP-I, n=67).

Fertility index	MTC	PVP-I
Overall conception rate (%)	23/42 (54.76%)	29/67 (43.28%)
First service conception (%)	17/42 (40.47%)[a]	9/67 (13.43%)[b]
No of services per conception	1.4±0.02[a]	2.9±0.87[b]
Treatment-conception interval (days)	45.32±5.14[a]	112.36±27.89[b]

Values are presented as percentages or means±standard error. Values with the same superscript letter in the same column are not significantly different. Statistical significance was set at P<0.05.

According to the obtained results, the dendrogram exhibited that the analyzed *S. aureus* isolates were closely related to four reference strains of coagulase negative *S. aureus* and various from coagulase negative *staphylococcus hominis* in the Bruker taxonomy. Another close relation for *Acinetobacter junii* isolates was detected with three strains of *Acinetobacter junii*. Moreover, the MSP dendrogram exhibited a strong relationship between the field isolates of *Escherichia coli* and 5 reference strains in the library of Microflex Compass software at the distance level of 100. A weak relationship was illustrated between the identified *Corynebacterium* spp. and three reference strains in the Bruker library.

The primary peaks of the m/z ratios of the ribosomal proteins of the field isolates were analyzed and it was discovered that ribosomal proteins are important components in MALDI-TOF MS-based bacterial identification (Elbehiry *et al*., 2016).

The bacterial species that were found associated with subclinical endometritis in female dromedaries in the current study are not unexpected. *Staphylococcus aureus* was the

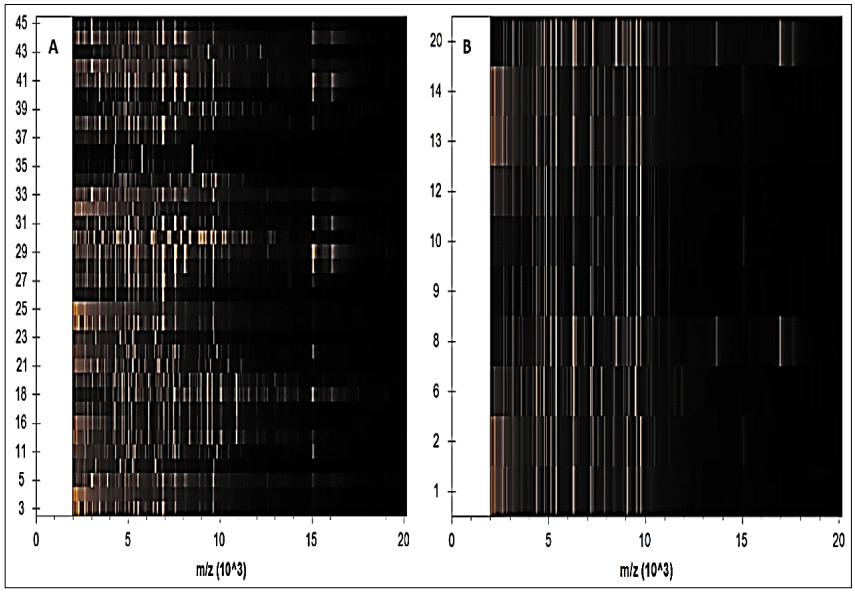

Fig 2: Gel view of protein spectra for 176 gram-positive (A) and 50 gram-negative (B) bacterial isolates. The yellow color of spots was the gathering of protein spectra with various contents.

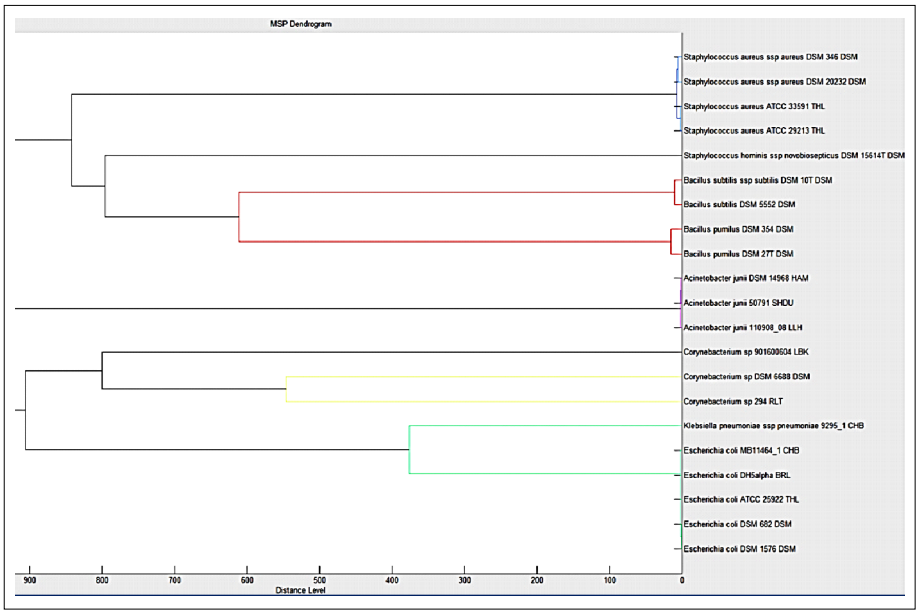

Fig 3: The MSP dendrogram for 176 gram-positive and 50 gram-negative bacteria exhibited a strong relation for *Staphylococcus aureus*, *Acinetobacter junii*, *Escherichia coli*, *Bacillus pumilus* and *Bacillus subtilis* field isolates in comparison with the reference strains in the Bruker taxonomy.

most frequently isolated bacterium, followed by *Escherichia coli*, *Corynebacterium* sp., *Bacillus pumilus*, *Acinetobacter junii*, *Bacillus subtilis* and *Klebsiella pneumonia*. These species were previously found associated with clinical or subclinical infections in the genital tract of female dromedaries (Mshelia *et al*., 2014; Wagener *et al*., 2014; Ali *et al*., 2015).

Despite the fact that the conception rate reported in the present study for both treatment protocols, MTC had the advantage over PVP-I due to the shorter treatment-conception interval, the higher percentage of animals conceived after first service and the lower number of services per conception. The post-treatment fertility indices found in the present study are in accordance with figures reported before for treatment protocols designed for female dromedaries affected with various degrees of uterine infections (Ali *et al*., 2010). PVP-I works originally via its iodine contents as a broad spectrum antibacterial agent. To treat uterine disorders that are caused by residual hormones or antibiotics, antibiotic-resistant bacteria, or withdrawal periods, safe and effective treatments are required. PVP-I, on the other hand, has the advantage of not requiring a withdrawal period and not passing into the milk except in the case of excessive dosing (Carleton *et al*., 2008). PVP-I acts as an astringent on healthy mucous membranes while having no effect on viability. Bacteria, fungi, yeasts and protozoa all have cell walls that iodine destroys directly. As a result, PVP-I action is not pathogen specific, but it can target a wide range of pathogens (Mido *et al*., 2016).

When it comes to using MTC to treat bacterial illnesses, it's important to note that bacterial cultures and antibiotic susceptibilities are the most effective ways to tackle the problem of antibacterial selection. Furthermore, what tissues are implicated in the uterine infection that is being treated? Systemic therapy may be required if the infection has spread to deeper layers of the uterus and other genital organs. If the infection is limited to the endometrium, however, local therapy is likely required due to the presence of very high-sustained antibiotic levels in the lumen and endometrium (Ali *et al*., 2010). The two treatment protocols in this investigation included a prostaglandin ($PGF_2\alpha$) analogue. We hypothesized that it has a therapeutic impact on the female camel's genital system, similar to what has been observed in cattle. $PGF_2\alpha$ produces luteolysis of a responsive corpus luteum (CL) in cyclic cows, which results in lower progesterone levels and subsequent estrus, as well as higher estrogen levels and myometrial contractions. All of these events appear to be favorable for uterine infection clearance (Weems *et al*., 2006). Furthermore, earlier research in cattle has suggested that $PGF_2\alpha$ has direct effects on the uterus (LeBlanc *et al*., 2002) and that it can treat uterine infections in cows without a CL (Gilbert, 2004). Exogenously administered $PGF_2\alpha$ has been demonstrated to increase uterine $PGF_2\alpha$ and luteal leukotriene B4 (LTB4) production, as well as promote chemotaxis and antibody-independent cell-mediated cytotoxicity (Hoedemaker *et al*., 1992). $PGF_2\alpha$ is also a proinflammatory molecule that can trigger proinflammatory cytokines that improve phagocytosis and lymphocyte activities (Kelly *et al*., 2001).

CONCLUSION

Based on the present data, it can be concluded that MALDI-TOF is efficient, fast and reliable for the detection and identification of various bacterial agents incriminated in uterine infections in female dromedary. Fertility indices in treated female dromedaries associated with subclinical

endometritis favors the use of intrauterine infusion of cephapirin benzathine therapy in the present study.

Compliance with ethical standards

The Animal Care and Welfare Committee of the Deanship of Scientific Research at Qassim University in the Kingdom of Saudi Arabia gave their approval to this work (Number 213372).

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Data availability

Data is available upon reasonable request from the authors of the manuscript

Conflict of interest: None.

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