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J. Clin. Microbiol. 2014, 52(5):1658. DOI: 10.1128/JCM.03032-13.

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Identification of Coagulase-Negative Staphylococci from Bovine Intramammary Infection by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry

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Coagulase-negative staphylococci (CoNS) are among the main pathogens causing bovine intramammary infection (IMI) in many countries. However, one of the limitations related to the specific diagnosis of CoNS is the lack of an accurate, rapid, and convenient method that can differentiate the bacterial species comprising this group. The aim of this study was to evaluate the ability of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) to accurately identify CoNS species in dairy cow IMI. In addition, the study aimed to determine the frequency of CoNS species causing bovine IMI. A total of 108 bacterial isolates were diagnosed as CoNS by microbiological cultures from two milk samples collected from 21 dairy herds; the first sample was collected at the cow level (i.e., 1,242 composite samples from all quarters), while the second sample was collected at the mammary quarter level (i.e., 1,140 mammary samples collected from 285 cows). After CoNS isolation was confirmed by microbiological culture for both samples, all CoNS isolates ($n = 108$) were genotypically differentiated by PCR restriction fragment length polymorphism (RFLP) analysis of a partial *groEL* gene sequence and subjected to the MALDI-TOF MS identification procedure. MALDI-TOF MS correctly identified 103 (95.4%) of the CoNS isolates identified by PCR-RFLP at the species level. Eleven CoNS species isolated from bovine IMI were identified by PCR-RFLP, and the most prevalent species was *Staphylococcus chromogenes* ($n = 80$; 74.1%). In conclusion, MALDI-TOF MS may be a reliable alternative method for differentiating CoNS species causing bovine IMI.

The progress in taxonomy for further identification of *Staphylococcus* species has been a lengthy process. In previous classification schemes, coagulase-positive organisms were categorized as *Staphylococcus aureus*, while coagulase-negative organisms were classified as *Staphylococcus epidermidis* or *Micrococcus* spp. (1). The organization of *Staphylococcus* spp. into biotypes (2) and further studies performed in the 1970s and 1980s led to the introduction of new species and subspecies of coagulase-negative staphylococci (CoNS) (3–7).

Currently, coagulase-negative staphylococci are the most prevalent microorganisms causing mastitis, especially in dairy herds where primary mastitis pathogens have been controlled as a result of specific treatment and prevention programs (8). Sixteen CoNS species have been previously isolated from cows with clinical and subclinical mastitis (9). Cows with mastitis caused by *S. chromogenes*, *S. simulans*, and *S. xylosus* had somatic cell counts (SCC) similar to those observed in cows with mastitis caused by *S. aureus* (10). Additionally, *S. chromogenes* and *S. epidermidis* seem to be more adapted to the mammary gland than other species (*S. equorum*, *S. sciuri*, *S. fleuretti*, *S. cohnii*, *S. devriesei*, *S. xylosus*, *S. arlettae*, and *S. succinus*) which have shown patterns of environmental transmission (3, 11–14). Thus, CoNS identification at the species level is important due to the epidemiologic and pathogenic differences between the various CoNS species causing mastitis.

Mastitis diagnosis programs still lack a rapid and reliable identification method that can discriminate between CoNS species isolated from bovine intramammary infections (IMI). Conventional procedures for the differentiation of CoNS species are based on phenotypic tests, which are considered labor-intensive and time-consuming (15). Moreover, miniaturized panels of bio-

chemical tests are considered expensive and are not accurate for the identification of *Staphylococcus* spp. in samples collected from animals (16, 17). Molecular biology techniques offer advantages due to their more rapid speed and specificity for the identification of microorganisms (18). However, to date, no standard methodology has been widely accepted for identifying causative agents of mastitis by genotypic patterns.

The method of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is based on the differentiation of protein profiles from microorganisms and has been used for clinical microbiological diagnosis in human medicine (19, 20). MALDI-TOF MS is considered a fast, accurate, and species-specific identification method (21–23); however, few studies have evaluated this methodology for the identification of microorganisms causing bovine IMI (24). A recent study evaluating the technique of MALDI-TOF MS as an alternative method for identification of microorganisms causing bovine mastitis identified only five CoNS species from 33 milk samples (24). However,

Received 29 October 2013 Returned for modification 16 December 2013

Accepted 3 March 2014

Published ahead of print 12 March 2014

Editor: B. A. Forbes

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doi:10.1128/JCM.03032-13

TABLE 1 Reference strains used as controls for the MALDI-TOF MS and PCR-RFLP analyses

Species	Strain
<i>S. aureus</i>	ATCC 12600
<i>S. capitis</i>	ATCC 27840
<i>S. caprae</i>	ATCC 35538
<i>S. chromogenes</i>	ATCC 43764
<i>S. cohnii</i>	ATCC 29974
<i>S. epidermidis</i>	ATCC 14990
<i>S. haemolyticus</i>	ATCC 29970
<i>S. hominis</i>	ATCC 27844
<i>S. hyicus</i>	ATCC 11249
<i>S. saprophyticus</i>	ATCC 15305
<i>S. sciuri</i>	ATCC 29061
<i>S. simulans</i>	ATCC 27848
<i>S. warneri</i>	ATCC 10209
<i>S. xyloso</i>	ATCC 29971

the latter study did not aim to specifically identify CoNS species that were potentially causing mastitis.

The aims of the present study were to evaluate the technique of MALDI-TOF MS for the differentiation of CoNS species isolated from dairy cows with mastitis and to determine the frequency of isolation of CoNS species causing bovine mastitis.

MATERIALS AND METHODS

Sample collection and bacterial strains. Two milk sample collections were performed by selecting cows with IMI caused by CoNS. In the first sample collection, composite milk samples were collected from all lactating cows ($n = 1,242$) distributed among 21 dairy herds located in the state of Sao Paulo, Brazil. Composite milk samples underwent microbiological culture (25) to screen for cows with CoNS IMI. After obtaining the culture results, we performed a second sample collection within a period of 15 days, and milk samples were collected from each mammary quarter of 285 dairy cows, for a total of 1,140 milk samples. A total of 108 CoNS isolates were identified by microbiological culture based on colony morphology, Gram staining, catalase testing, and coagulase testing (25). An IMI was diagnosed when both milk samples collected from the same cow during consecutive sample collections were microbiologically positive for CoNS. All CoNS isolates were maintained at -20°C in 1 ml of brain heart infusion broth (BHI) (BBL) supplemented with 10% glycerin until analysis by MALDI-TOF MS and PCR-restriction fragment length polymorphism (RFLP). Samples with more than two morphologically distinct bacterial isolates ($n = 16$) were considered contaminated and excluded from further analysis. Fourteen reference strains of CoNS were included in the study as positive controls (Table 1).

DNA extraction and precipitation for genotypic identification. Prior to DNA extraction, all isolates were cultured overnight on blood agar plates at 37°C under aerobic conditions to evaluate the purity of each CoNS isolate. The extraction of genomic DNA from pure cultures of CoNS isolates was performed with an Illustra bacteria genomicPrep mini spin kit (GE Healthcare, United Kingdom). Extracted bacterial DNA was precipitated by two ethanol washes (26). Briefly, nucleic acids were first washed using 500 μl of absolute ethanol and 20 μl of 3 M sodium acetate (pH 5.2) and incubated at -20°C for 12 h. The solution was then centrifuged at $23,000 \times g$ for 35 min at 4°C , and the pelleted DNA was washed with 500 μl of 70% ethanol and centrifuged at $7,500 \times g$ for 5 min. The ethanol was discarded, and the tubes were allowed to dry upside down for 15 min. The precipitated DNA was resuspended in 40 μl of Tris-EDTA (TE) buffer. The quality of the extracted DNA was assessed by measuring the absorbances at 260 nm and 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), and the DNA samples were stored at -20°C until PCR amplification.

PCR amplification. The *groEL* degenerate primers used to amplify a 550-bp *groEL* gene fragment included primer H279A (5'-GAIIGCIGGI GA(TC)GGIACIACIAC-3') and primer H280A [5'-(TC)(TG)I(TC)(TG)ITCICC(AG)AAICCGGIGC(TC)TT-3'] (27). The PCRs were performed in a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA) in a final volume of 50 μl containing 100 ng DNA, 0.5 μg of each primer, 250 μM each deoxyribonucleoside triphosphate, 1.5 mM MgCl_2 (Invitrogen Life Technologies, Brazil), and 2 U of *Taq* DNA polymerase (Invitrogen Life Technologies) in $1 \times$ amplification buffer (50 mM KCl and 20 mM Tris-HCl [pH 8.4]) (Invitrogen Life Technologies). The PCR mixtures were denatured (3 min at 95°C), subjected to 40 cycles of amplification (1 min denaturation at 94°C , 2 min annealing at 53°C , and 1 min elongation at 72°C) and completed with 1 cycle of 10 min at 72°C . The presence of a PCR product was confirmed by agarose gel electrophoresis.

PCR-RFLP. The CoNS species were genotypically identified using PCR restriction fragment length polymorphism (RFLP) analysis of a partial *groEL* gene sequence (15). The restriction fragment length polymorphism analysis used the *AluI* restriction endonuclease and was carried out as described by Santos et al. (15). Briefly, digestions were performed with 500 ng of PCR product in a total volume of 30 μl with $1 \times$ reaction buffer, 2 μg of acetylated bovine serum albumin, and 5 U of *AluI* (Promega, Madison, WI) for 12 h at 37°C . The resulting fragments were separated by electrophoresis through 10% polyacrylamide gels for 1 h and 40 min at 150 V in Tris-borate-EDTA buffer (pH 8.2). The gels were stained with ethidium bromide and visualized on a UV transilluminator. A double digestion with the restriction enzymes *HindIII* (10 U) and *PvuII* (10 U) was also performed following the same protocol for the identification of the species *S. chromogenes*, *S. hyicus*, and *S. capitis*. The size of the digested bands was determined by measuring the distance from the center of the well to the center of the band and comparing it to a 25-bp DNA ladder (Invitrogen Life Technologies). Also, the sizes of the digested bands from CoNS IMI were compared to the sizes of the digested bands from reference strains.

Sample preparation for MALDI-TOF MS. Sample preparation for MALDI-TOF MS was performed as previously described (24). Briefly, a few (2 to 4) CoNS colonies from a fresh and pure overnight culture grown on blood agar at 37°C under aerobic conditions were suspended in 300 μl distilled water, to which 900 μl absolute ethanol was added. The resulting solution was homogenized and centrifuged at $13,000 \times g$ for 2 min, and the supernatant was discarded. A solution of 70% formic acid (30 μl) was added to the pellet to lyse the bacterial cells and release their intracellular proteins, most critically, the ribosomal proteins (28). Subsequently, an equal volume of 100% acetonitrile was added to each tube, thus producing a bacterial extract in a 1:1 solution of 70% formic acid and acetonitrile. A final centrifugation step ($13,000 \times g$ for 2 min) was performed to separate bacterial cell debris from the supernatant containing the intracellular proteins used for the bacterial identification.

MALDI-TOF MS. Samples were processed in an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with flex control software (Bruker Daltonics) operated in linear mode and equipped with a 337-nm nitrogen laser. To prepare the MALDI target plate, 1 μl of each bacterial extract was spotted onto a 384-spot target plate (polished stainless steel, Bruker Daltonics) and dried at room temperature. The dried sample spot was overlaid with 1 μl of a matrix solution, consisting of α -cyano-4-hydroxy-cinnamic acid diluted in a solution of 50% acetonitrile and 2.5% trifluoroacetic acid. The Bruker bacterial test standard (BTS) (Bruker Daltonics) was used for mass calibration and for instrument parameter optimization. Mass spectral data were collected within the m/z range of 2,000 to 20,000, and the data were acquired using the FlexControl software 3.3 (Bruker Daltonics).

Data analysis. Biotyper 3.0 software (Bruker Daltonics) was used to process the raw spectra acquired by the Autoflex. Data were analyzed using the built-in main spectra projection feature of the Biotyper software, which is a proprietary algorithm for spectral pattern matching that produces a logarithmic score from 0 to 3. The peak lists were compared

TABLE 2 Frequency of distribution of coagulase-negative staphylococci identified by PCR-RFLP versus MALDI-TOF MS

CoNS species (as identified by PCR-RFLP)	No. of isolates	MALDI-TOF MS		
		Identification level		CI ^c (%)
		Genus ^a	Species ^b	
<i>S. chromogenes</i>	80	0	80	100
<i>S. saprophyticus</i>	6	4	2	33.33
<i>S. haemolyticus</i>	5	1	4	80
<i>S. simulans</i>	4	0	4	100
<i>S. xyloso</i>	3	0	3	100
<i>S. aureus</i> coagulase negative	3	0	3	100
<i>S. capitis</i>	2	0	2	100
<i>S. epidermidis</i>	2	0	2	100
<i>S. sciuri</i>	1	0	1	100
<i>S. cohnii</i>	1	0	1	100
<i>S. hyicus</i>	1	0	1	100
Total	108	5	103	95.37

^a Scores produced by Biotyper 3.0 were ≥ 1.7 and < 2 .^b Scores produced by Biotyper 3.0 were ≥ 2 .^c CI, correctly identified rate.

with each entry in the Biotyper database, which contained 3,995 references, using the standard parameters of the pattern-matching algorithm. The identification score cutoff values were applied to each measurement according to the manufacturer's instructions. Scores of ≥ 2 indicate species-level identification, scores of ≥ 1.7 but < 2 indicate genus-level identification, and scores of < 1.7 indicate that no reliable identification can be made. The CoNS species-specific identification provided by MALDI-TOF MS was compared with the diagnostic results obtained by PCR-RFLP, thus providing the frequency of diagnosis equivalence between the two methods.

RESULTS

All isolates identified as CoNS after two microbiological cultures underwent species-level identification using PCR-RFLP, which identified 11 different species (Table 2). The MALDI-TOF MS method correctly identified, at the species level, 103 (95.37%) of the 108 CoNS isolates identified by PCR-RFLP. Two isolates (1.85%) identified by PCR-RFLP as *S. saprophyticus* and *S. haemolyticus* were identified as the correct species by MALDI-TOF MS, but both had scores of ≥ 1.7 and < 2 . The results for three isolates (2.78%) did not match between the two diagnostic methods used in this study. These three isolates were identified as *S. saprophyticus* by PCR-RFLP and as *S. aureus* ($n = 2$) and *S. hominis* ($n = 1$) by MALDI-TOF MS, with scores of ≥ 1.7 and < 2 . Three isolates of coagulase-negative *S. aureus* as identified by PCR-RFLP were identified as *S. aureus* by MALDI-TOF MS, with scores of ≥ 2 (Table 2). All reference strains ($n = 14$) identified by PCR-RFLP matched the identification by MALDI-TOF MS.

Among the CoNS isolates ($n = 108$) identified by PCR-RFLP to the species level, 80 (74.07%) were identified as *S. chromogenes*, which was the species with the highest frequency of identification, followed by *S. saprophyticus* ($n = 6$; 5.55%) and *S. haemolyticus* ($n = 5$; 4.63%). Three isolates (2.78%) were identified as coagulase-negative *S. aureus*. Other CoNS species identified by PCR-RFLP included *S. simulans* ($n = 4$), *S. xyloso* ($n = 3$), *S. capitis* ($n = 2$), *S. epidermidis* ($n = 2$), *S. sciuri* ($n = 1$), *S. cohnii* ($n = 1$), and *S. hyicus* ($n = 1$) (Table 2).

DISCUSSION

The ample variety of CoNS with the potential to cause bovine mastitis makes diagnosis at the species level difficult (14). Coagulase-negative staphylococci species identification is also limited by the lack of a simple and rapid diagnostic method that can reliably differentiate the species in this group of microorganisms (29). In the present study, the results of CoNS species identification from bovine IMI samples by MALDI-TOF MS and PCR-RFLP were in agreement for 95.37% of the isolates. The results from our study indicate that MALDI-TOF MS can be a rapid and accurate species-specific method for microorganism diagnosis. Rapid diagnosis enables better decisions regarding herd management or antimicrobial treatment of mastitis, thus preventing or reducing the harmful effects of intramammary infections caused by potentially contagious CoNS species (13).

In this study, we used PCR-RFLP of the *groEL* gene as the reference method for identification of CoNS species from dairy cow IMI. The method of PCR-RFLP of the *groEL* gene was compared with the reference biochemical method (30, 31) and identified 100% of CoNS species isolated from human clinical samples (32) and from milk samples of dairy cows with IMI (15).

The *groEL* gene has been suggested to be an ideal universal DNA target for identification of CoNS to the species level because it is ubiquitous and encodes a highly conserved and essential housekeeping protein (15). This gene has well-conserved DNA sequences within a given species but with sufficient sequence variations to allow species-specific identification (33). A number of PCR amplicon sequencing-based methods targeting the 16S rRNA, *sodA*, and *tuf* genes have been reported for identification of CoNS (9, 16, 34). However, a drawback of sequence-based genotypic methods may be a lack of quality of deposited sequences in the GenBank database, especially for identification of closely related *Staphylococcus* species (35).

A recent study compared five methods for the identification of CoNS species from human clinical cultures (36): Vitek2 (bioMérieux), the ID 32 Staph strip (bioMérieux), partial 16S rRNA gene sequencing (MicroSeq; Applied Biosystems), partial *tuf* gene sequencing, and MALDI-TOF MS (Bruker Daltonics). A higher identification rate (99.3%) was reported for MALDI-TOF MS in this study than for the ID 32 Staph strip (85.9%), Vitek2 (92.3%), partial 16S rRNA gene sequencing (70.4%), and partial *tuf* gene sequencing (93%). The identification rate of CoNS species provided by MALDI-TOF MS observed in our study was greater than the identification rate observed for the genotypic methods evaluated by Loonen et al. (36), which suggests that MALDI-TOF MS can be a reliable alternative method for the differentiation of CoNS species causing bovine IMI.

However, the method of MALDI-TOF MS has some limitations that must be considered. Identification of microorganisms is possible only if the spectrum related to the strain of interest is recorded in the database of the software Biotyper. Additionally, calibration of equipment and adjustments to the internal patterns are necessary steps when seeking the intraspecific characterization of microorganisms (19). Another limitation of MALDI-TOF MS is related to the difficulty of differentiating pathogens present in mixed cultures due the generation of overlapping spectra (37). Furthermore, the high initial acquisition cost of a MALDI-TOF mass spectrometer may limit the use of this method to the identification of pathogens causing mastitis on a commercial level.

According to the proposed acceptance criteria for the identification of *Staphylococcus* species, the score cutoff values for CoNS isolates ideally need to be ≥ 2 (36). In this study, when only those CoNS isolates with scores of ≥ 2 were considered, the results of MALDI-TOF MS and PCR-RFLP were in agreement for 98.09% of the samples. For studies that evaluated the method of MALDI-TOF MS for identification of CoNS species isolated from human clinical samples, the reported correct identification rates ranged from 74.2% to 99.3% (36). The variability of CoNS identification rates observed among studies might be explained by differences in the conditions of bacterial growth, preparation of samples, number of reference strains, version of the software Biotyper, and design of the studies (19).

All CoNS isolates that had no identification agreement ($n = 3$) between the two methods used in this study were identified by PCR-RFLP as *S. saprophyticus*. After analysis by MALDI-TOF MS, these three isolates had identification scores of ≥ 1.7 and < 2 , which indicates a reliable identification at the genus level (i.e., *Staphylococcus* spp.). Misidentification by MALDI-TOF MS has been reported for *S. saprophyticus* identification when the standard extraction method (i.e., the formic acid method) was used (22). Additionally, the microorganism identification by MALDI-TOF MS is dependent on a good ribosomal protein extraction from the bacteria (38). Thus, the extraction procedure used in our study may not have released a sufficient amount of ribosomal protein for reliable species identification of the three isolates identified at the genus level by MALDI-TOF MS.

Another reasonable explanation for the species misidentification by MALDI-TOF MS in this study might be the absence of subspecies associated with bovine mastitis in the database of the Biotyper software. An essential step in the identification of bacteria at the species level by MALDI-TOF MS has been the use of dedicated databases with rigorous data quality control and powerful algorithms for comparison with mass fingerprinting (38). The Biotyper database has been primarily developed for human clinical microbiology and has recently come into use for identification of veterinary isolates. The depth of the spectral database for veterinary isolates is still sporadic but has evolved with each database update.

Eleven CoNS species were isolated from bovine IMI in this study, a result which indicates an ample variety of CoNS species potentially causing IMI in dairy cows. According to Capurro et al. (9), 16 CoNS species have been isolated from milk samples collected from dairy cows with mastitis. *Staphylococcus chromogenes* was the CoNS species most frequently isolated (74.07%) from dairy cows with IMI in this study. Other studies have also reported *S. chromogenes* as the most prevalent species isolated in milk samples from cows with mastitis caused by CoNS (3, 39–43), although with a lower frequency of isolation than observed in the present study.

Staphylococcus chromogenes seems to be the CoNS species most adapted to the bovine mammary gland because it is not frequently isolated from extramammary sites (14, 42). Piessens et al. (41) reported a high similarity between genotypes of *S. chromogenes* isolated from milk samples and suggested that specific genotypes of this species are well adapted to the mammary gland, which is typically observed for contagious udder pathogens. Consequently, contagious transmission among cows seems to be an important route for new IMI caused by *S. chromogenes*. Another possible reason for the high prevalence of *S. chromogenes* is its resistance to

antibiotics (44) and its ability to persist in the mammary gland. Taponen et al. (45) reported that 54.5% of IMI caused by *S. chromogenes* persisted throughout lactation. Additionally, *S. chromogenes* was identified as the CoNS species with the lowest sensitivity to the teat disinfectants most commonly used for milking management (46). Factors such as the adaptability to mammary gland associated with the potential for infection persistence, reduced sensitivity to teat disinfectants, and antibiotic resistance may explain the high prevalence of *S. chromogenes* isolated from dairy cows in this study. Although *S. chromogenes* was the most frequently isolated CoNS species from dairy cows with IMI in this study, further studies are needed to substantiate the prevalence of this species in bovine mastitis at the country level.

The other species of CoNS ($n = 10$) isolated in this study accounted for only 25.93% ($n = 28$) of all isolates identified by PCR-RFLP. The other species of CoNS identified in this study have been previously reported with low frequencies of isolation in other studies evaluating cows with IMI caused by CoNS (13, 47, 48).

Three CoNS isolates were identified as coagulase-negative *S. aureus*. Fox et al. (47) also identified a variant of *S. aureus* with a negative coagulase test and suggested that the ability of *Staphylococcus* spp. to coagulate rabbit blood plasma is a virulence factor that can increase the milk SCC of dairy cows. Isolates identified as coagulase-negative *S. aureus* were considered members of the CoNS group because they belong to the genus *Staphylococcus* and react negatively on the coagulase test.

The results of this study suggest that MALDI-TOF MS could be an alternative method for identifying CoNS species causing bovine IMI. Species of CoNS, especially *S. chromogenes*, have shown contagious and pathogenic profiles similar to those of *S. aureus* (10), which indicates the importance of a rapid and accurate microbiological diagnosis. Considering the difficulty of CoNS species differentiation, in part due to the diversity of species potentially causing IMI, MALDI-TOF MS can be a reliable and fast method to differentiate between CoNS species causing mastitis.

In conclusion, MALDI-TOF MS combined with the software Biotyper 3.0 is an alternative method for CoNS species identification from samples isolated from dairy cows with IMI. Furthermore, based on the prevalence of CoNS species isolated from milk samples collected from dairy cows with IMI, *S. chromogenes* is the most frequently isolated microorganism.

ACKNOWLEDGMENTS

We are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil, for a scholarship award (2011/04063-1) and research funding (2011/14284-5).

We also thank Márcia Giambiagi de Marval and Elaine Menezes Barros (Department of Medical Microbiology, Federal University of Rio de Janeiro, Brazil) for their assistance with the PCR-RFLP analysis.

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