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DETECTION OF THE FILARIAL PARASITE *MANSONELLA STREPTOCERCA* IN SKIN BIOPSIES BY A NESTED POLYMERASE CHAIN REACTION–BASED ASSAY

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Abstract. To differentiate the skin-dwelling filariae *Mansonella streptocerca* and *Onchocerca volvulus,* a nested polymerase chain reaction (PCR) assay was developed from small amounts of parasite material present in skin biopsies. One nonspecific and one specific pair of primers were used to amplify the 5S rDNA spacer region of *M. streptocerca.* Biopsies with different microfilaria densities obtained from 104 Ugandans living in an area endemic for *M. streptocerca* were tested using both the nested PCR assay and standard parasitologic assessment of microfilariae. All 82 samples from microfilaria carriers were positive when tested using the nested PCR assay. In addition, *M. streptocerca* DNA could be detected in 16 samples thought to be microfilaria negative. Furthermore, six days following ivermectin treatment, *M. streptocerca* DNA was found in 12 of 14 microfilaria-negative biopsies. Control skin samples from patients infected with *O. volvulus* were all negative in the nested PCR assay. This assay improves the diagnosis of *M. streptocerca* and will facilitate further epidemiologic studies.

When evaluating skin disease cases in large regions of Africa, the two skin-dwelling filarial species, *Mansonella streptocerca* and *Onchocerca volvulus,* must be considered as possible causative agents. *Mansonella streptocerca* infection is characterized by acute and more often chronic papular dermatitis mainly on the upper part of the body, which make it difficult to differentiate streptocerciasis and onchocerciasis clinically.[1, 2] In addition, after treatment with diethylcabarmazine or ivermectin, persons infected with *M. streptocerca* also show a typical Mazzotti reaction.[1, 3] Cross-reactions between *O. volvulus* and *Mansonella* species impede the differentiation of these parasites by serologic diagnosis using worm extracts.[4] Although isolated microfilariae (mf) of both species can be differentiated morphologically, species differentiation in histologic sections is difficult. Since the mf densities of *M. streptocerca* are often relatively low, methods other than the standard skin snip technique are required for accurate detection of this species. Collagenase digestion of the skin snips is an appropriate procedure,[2] but if a patient is also heavily infected with *O. volvulus* it may be difficult to detect one *M. streptocerca* mf among hundreds of *O. volvulus* mf. In these cases, a specific but more sensitive assay to detect *M. streptocerca* is clearly required.

The polymerase chain reaction (PCR) is a sensitive technique to detect parasite DNA. Sensitive and specific PCR assays have been developed for the diagnosis of the human filarial parasites *Wuchereria bancrofti, Brugia malayi, Loa loa,* and *O. volvulus.*[5–10] All of these assays are based on different repetitive target sequences found only within a distinct filarial genus. In the present study, we have developed a PCR assay based on a target sequence present in all filarial and all other nematode species.[11] The 5S rDNA spacer region was amplified from DNA prepared from human skin biopsies that contained only a small number of *M. streptocerca* mf. The primers used in the assay are based on conserved coding regions of the 5S rDNA. After sequencing of the 5S rDNA spacer PCR product, species-specific primers were designed to detect *M. streptocerca* specifically in a nested PCR assay. The sensitivity and specificity of this assay was evaluated on clinical samples collected in an endemic area in western Uganda.

MATERIALS AND METHODS

Patient samples. Skin biopsies were collected from persons living in the Bundibugyo district located north of the Ruwenzori mountains in western Uganda.[2] This region is endemic for *M. streptocerca,* but not for *O. volvulus.* For the evaluation of mf density, each biopsy was placed into a single well of a microtiter plate containing phosphate buffer and was incubated overnight at room temperature. The solution was than pipetted onto a glass slide for microscopic examination using 63× magnification. Subsequently, the remaining biopsy material from each well was preserved in 80% ethanol and stored in a refrigerator for later PCR testing. For comparison, skin snips were collected from patients living in an area of western Uganda endemic for *O. volvulus,* but not for *M. streptocerca.*[12] Genomic *O. volvulus* DNA was also prepared from female worms isolated from extirpated nodules from patients in the same area. In both the *M. streptocerca*- and *O. volvulus*-endemic regions, the blood filarial parasite *Mansonella perstans* was coendemic. No other filarial species infecting humans were found in these regions.[2, 13] Single specimens from filariasis patients of other geographic regions were also available from previous studies. Informed consent was obtained from all individuals who participated in the investigation. The study was approved by the Ministry of Health of Uganda and by the Ethics Commission of the Medical Board of Hamburg, Germany.

All persons were examined for filarial skin lesions before and six days following ivermectin treatment according to the recommendations by Murdoch and others[14] and the World Health Organization.[15] The skin reaction of microfilaria carriers following administration of microfilaricidal drugs (Mazzotti reaction) was used as an additional tool to determine infection. Only fresh papules seen by the examiner following treatment were recorded as a positive Mazzotti test result. Most patients presented a history of increased itching following treatment. Since such a statement cannot be verified by the examiner, it was not used as a criterion for a positive Mazzotti test result. All consenting persons were treated with a single oral dose of ivermectin (150 μg/kg of body weight), except three who presented with pregnancy,

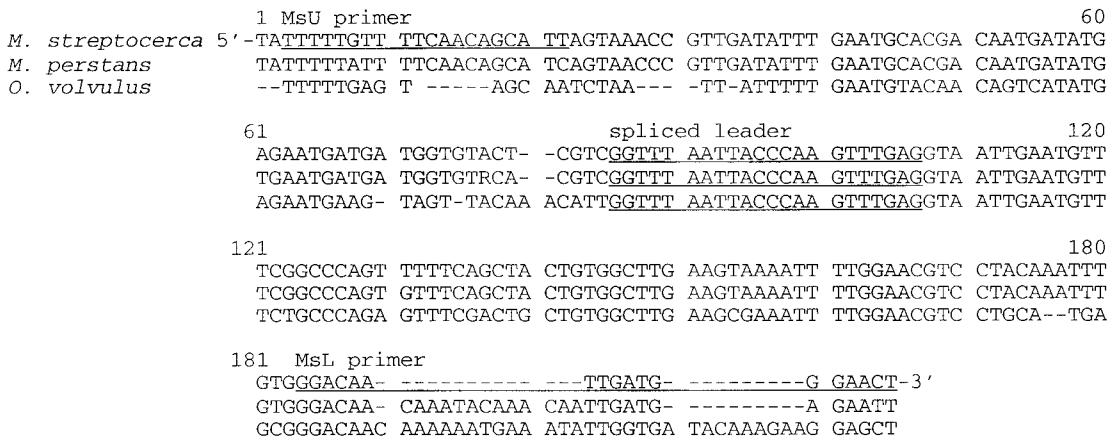

FIGURE 1. Sequence of the 5S rDNA spacer region, the location of the second set of primers (MsU and MsL), and the location of the spliced leader sequence in *Mansonella streptocerca, M. perstans,* and *Onchocerca volvulus.*

asthma, and a doubtful neurologic disorder. A few patients did not come for the follow-up examination and some were only treated at the second visit. Therefore, 73 patients were re-examined for a Mazzotti reaction.

Sample preparation and PCR. The DNA was prepared from skin snips, microfilariae, and adult *O. volvulus* as described previously.[16] For some samples, a simplified sample preparation method was used. Fifty microliters of nucleolysis buffer (0.05 M NaCl, 0.01 M EDTA, 0.05 M Tris base) and 25 μl of 1 N NaOH were added to the samples and incubated for 1 hr at 60°C. The solution was then neutralized using 1 N HCl and the DNA was ethanol precipitated overnight at −20°C. The DNA was pelleted by centrifuging at 13,800 × *g* for 20 min, dried and resuspended in 50 μl of Tris-EDTA buffer (0.5 M Tris base, 0.05 M EDTA). Two microliters of each extraction were subjected to the first PCR. During the DNA preparation and the PCR, standard precautions were applied to prevent contamination. Skin snips from areas nonendemic for *M. streptocerca* functioned as negative controls during the DNA preparation procedure and all mf negative samples were tested twice in the nested PCR assay. Unless specifically noted, all chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany). All oligonucleotides used in this study were custom syntheses from Integrated DNA Technologies, Inc. (Coralville, IA).

The PCRs were performed using *Taq* Gold Polymerase (Perkin Elmer, Norwalk, CT). The PCR mixture contained 1× PCR Buffer II (Perkin Elmer), 1.5 mM $MgCl_2$, 500 nmol of each dNTP, 20 pmol of each primer, and 2 U/50 μl of *Taq* Gold Polymerase. The primers were S2 5'-GTTAAGCAACGTTGGGCCTGG-3' and S16 5'-TTGACAGATCGGACGAGATG-3'.[11] The thermocycling program started with a hot start of 10 min at 94°C to activate the the *Taq* Gold polymerase, followed by 35 or 25 cycles of denaturation for 1 min at 90°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C; all were followed by a final extension for 5 min at 72°C (DNA Engine: MJ Research Inc., Watertown, MA). Eight microliters of the PCR product of each sample were separated by electrophoresis on an 1.5% agarose gel using TAE electrophoresis buffer (0.04 M Tris-acetate, 0.001 M EDTA) and visualized with ethidium bromide/UV illumination. For the second PCR, the same cycling conditions were chosen except that only 1 μl of the PCR product was used as template and only 25 cycles were performed. The primers used in the second PCR are shown in Figure 1.

Sequencing of DNA and nested PCR primer design. Dideoxy sequencing of PCR products was performed by direct cycle sequencing using one of the PCR primers as a sequencing primer. First, the PCR products were purified using silica-gel membrane spin-columns (Qiaquick PCR Purification Kit; Qiagen, Hilden, Germany). Fluorescence-based sequencing was performed using the ABI Prism DNA Sequencing Kit following the protocol of the manufacturer (Applied Biosystems Division, Perkin Elmer, Foster City, CA). Unincorporated dideoxy terminators were removed using Centri-Sep columns (Princeton Separation, Adelphia, NJ). Subsequently, the sequencing reactions were run on an automated sequencer (ABI 373A; Perkin Elmer). Results were further analyzed using the programs Seq Ed version 1.0.3 (ABD; Perkin Elmer) and Oligo 5.0 (National Bioscience Inc., Plymouth, MN) run on Macintosh (Cupertino, CA) Power PC computers. The two designed internal primers (MsU and MsL) had sizes of 20 and 18 basepairs (bp), respectively. They had melting temperatures of 58.5°C and 56.1°C (at concentrations of 100 pM and 1,000 mM Na^+), respectively.

RESULTS

Sequence analysis of the 5S rDNA spacer region. In the first PCR using the conserved S2 and S16 primers, the 5S rDNA spacer region of *M. streptocerca* was amplified from nine of 11 skin biopsies obtained from persons living in western Uganda. The size of the PCR products was approximately 420 bp (Figure 2). Six of these PCR products were sequenced using direct cycle sequencing. The sequence identity among these six samples was about 98%. For comparison, PCR products from samples obtained from patients infected with *O. volvulus* or *M. perstans* were also sequenced. These sequences were almost identical to those published by Xie and others.[11] One hundred ninety-nine nucleotides of the amplified spacer sequence were used to compare *M. strep-*

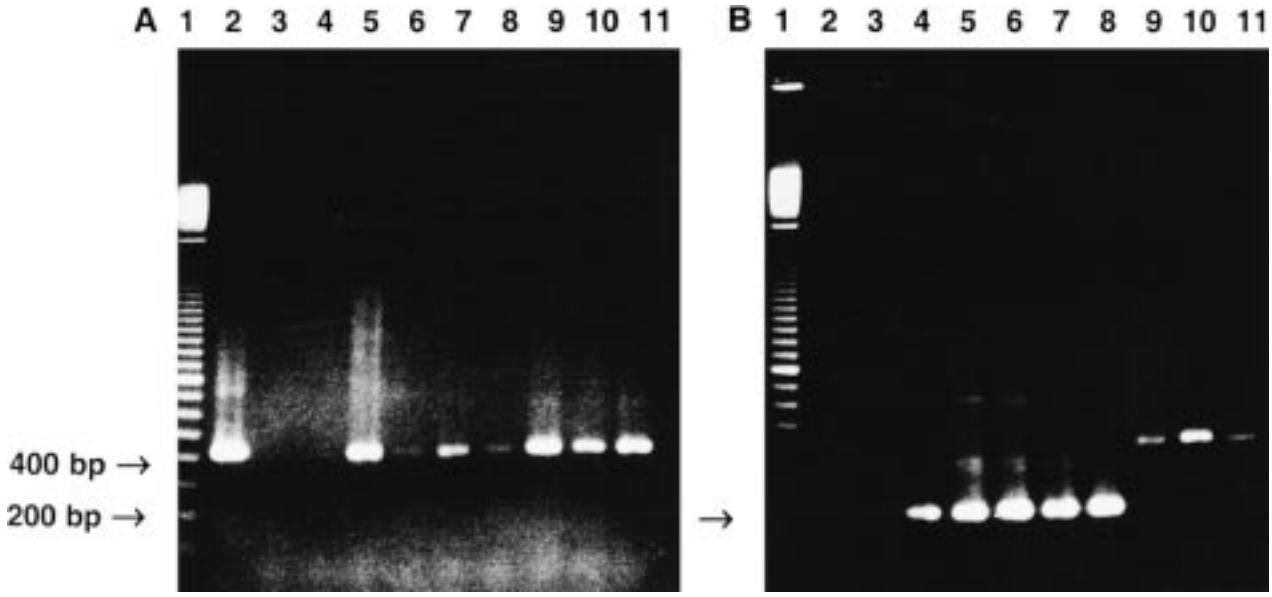

FIGURE 2. Detection of microfilariae (mf) in skin biopsies using a nested polymerase chain reaction (PCR)–based assay. Lane 1, 100 basepair (bp) ladder; lane 2, adult *Onchocerca volvulus* DNA; lane 3, negative control; lanes 4–8, skin biopsies from persons living in an area endemic for *Mansonella streptocerca* but not for *O. volvulus* (mf densities as determined by microscopic analysis of skin snips: 1.2, 0, 0.9, 1.0, and 6.2 mf/mg of skin, respectively); lanes 9–11, skin biopsies from persons living in an area endemic for *O. volvulus* but not for *M. streptocerca* (mf densities as determined by microscopic analysis of skin snips: 6.5, 0 and 26.7 mf/mg of skin, respectively). **A,** first PCR using the S2/S16 primers (yields a 400-bp PCR product). **B,** second PCR using the *M. streptocerca*-specific internal MsU/MsL primers (yields a 200-bp PCR product). The PCR product visible in lanes 9–11 are due to amplification of the 400-bp 5S rDNA spacer by residuary nonspecific primers (S2/S16).

tocerca, M. perstans, and *O. volvulus* (Figure 1). These comparisons revealed that *M. streptocerca* and *M. perstans* shared an identity of 90%, while *M. streptocerca* and *O. volvulus* showed 63% identity. All sequenced *M. streptocerca* samples showed a characteristic deletion of about 14 bp when compared with *M. perstans* and *O. volvulus* (Figure 1). However, a conserved region close to the 22-bp spliced leader sequence was found in all three species. The spliced leader sequence was, in all three cases, identical and was situated in the same plus strand orientation.

Nested PCR. The MsL/MsU primers generated a PCR product of about 200 bp (Figure 2B). The internal primers were tested at different annealing temperatures. The best results were achieved at an annealing temperature of 55°C. At 60°C, no detectable PCR product was obtained, while at 50°C (and below) the PCR was less specific and blood samples from patients infected with *M. perstans* were also positive. The most consistent results were obtained by performing 25 cycles in both the first and the second PCR assays. An increase in the number of cycles did not increase the sensitivity of the assay.

TABLE 1

Specificity of the nested polymerase chain reaction (PCR) for the detection of *Mansonella streptocerca* DNA in skin biopsies

Samples	No.	No. PCR positive: 1st PCR S2/S16 primers (non-specific)	No. PCR positive: 2nd PCr MsL/MsU primers (specific)
Onchocerca volvulus adults	3	3	0
O. volvulus skin snips	14	5	0
Wuchereria bancrofti microfilariae	1	1	0
Loa loa blood	1	1	0
O. volvulus/*M. perstans* area blood*	18	5†	0
M. streptocerca skin snips from Uganda	82	44	82
M. streptocerca skin snips from Ghana	5	0	4

* Blood samples obtained from individuals infected with *O. volvulus* and living in an area endemic for *M. perstans.*
† Thirty-five cycles.

To determine the specificity of the assay, various samples from filariasis patients or from persons living in endemic areas were tested (Table 1). After the first 25 cycles of PCR using the conserved primers (S2/S16), some samples containing DNA of *O. volvulus, W. bancrofti, L. loa,* or *M. perstans* were positive (i.e., gave a band of appropriate size on an ethidium bromide–stained gel). However, after the second, species-specific PCR using the MsL and MsU primers, none of these samples were positive. The results were in accordance with our expectations deduced from the DNA sequence comparisons. Since the assay was developed based on *M. streptocerca* samples collected from western Uganda, skin samples from patients living in an area in Ghana where *M. streptocerca* and *O. volvulus* are coendemic were also tested. *Mansonella streptocerca* DNA was detected in four of five biopsies from this region. This shows the applicability of the assay to detect *M. streptocerca* from different geographic regions.

The sensitivity of the nested PCR assay was tested on skin biopsies from Uganda with different *M. streptocerca* mf densities (0–209 mf/mg of skin). The mf densities were evaluated after overnight incubation as described in the Materials and Methods. *Mansonella streptocerca* mf emerged from 82 of the 104 tested skin snips. All of these mf-positive biopsies also tested positive in the nested PCR assay, indicating that *M. streptocerca* DNA was still present in all of the samples

TABLE 2

Sensitivity of the nested polymerase chain reaction (PCR) to detect *Mansonella streptocerca* DNA in skin biopsies from Ugandan patients using two different sets of primers and 25 cycles*

Mf density (mf/mg of skin)	No. of samples	No. PCR positive	
		1st PCR S2/S16 primer (%) (nonspecific)	2nd PCR MsL/MsU primer (%) (specific)
>4.9	35	23 (66)	35 (100)
1.0–4.9	29	12 (41)	29 (100)
0.1–0.9	18	5 (28)	18 (100)
0	22	4 (18)	16 (73)
Total	104	44 (42)	98 (94)

* Mf = microfilariae.

TABLE 3

Results of the nested polymerase chain reaction (PCR) assay compared with the occurrence of a Mazzotti reaction in 73 persons re-examined six days following treatment with a single dose ivermectin (150 μg/kg of body weight)*

Test result	Mazzotti reaction	
	No. of persons positive	No. of persons negative
mf positive/PCR positive	27	33
mf negative/PCR positive	4	4
mf negative/PCR negative	1†	4

* mf = microfilariae.
† Another biopsy from the right iliac crest of this individual revealed three mf and tested positive in the nested PCR assay.

(Table 2). Among the 22 mf-negative skin biopsies, 16 (73%) were positive in the nested PCR assay. Assuming that all individuals were infected, the sensitivity would be 94.2% (98 of 104). However, because it cannot be excluded that a few individuals were not infected with *M. streptocerca*, the sensitivity of the nested PCR assay is probably higher. These results demonstrate that the nested PCR assay is more sensitive than assessing the mf density by overnight incubation of skin snips for the diagnosis of *M. streptocerca* infection.

To prove whether *M. streptocerca* DNA is present following chemotherapy, 14 skin biopsies were tested from persons six days following treatment with a single dose ivermectin. Prior to treatment, all 14 of these biopsies tested positive by parasitologic assessment (0.4–54 mf/mg of skin) and by the nested PCR assay. Following the chemotherapy, no mf emerged from the biopsy, but 12 (86%) samples were still positive in the nested PCR assay.

None of the individuals who tested mf negative by skin snip assessment and negative in the nested PCR assay showed any skin conditions that could be related to *M. streptocerca* infection. In contrast, three of the 16 individuals who were mf negative but positive in the nested PCR assay, presented skin conditions consistent with *M. streptocerca* infection. These included papular dermatitis, scratch marks, and scars.

These results showed that the nested PCR assay is more sensitive than the microscopic assessment of mf in the skin. To investigate whether a Mazzotti reaction is caused by *M. streptocerca*, the occurrence of a Mazzotti reaction was compared with the result of the nested PCR assay before treatment (Table 3). Seventy-three of the 104 individuals were re-examined six days following chemotherapy with ivermectin to assess skin changes due to a Mazzotti reaction. Only one patient showed a positive Mazzotti reaction and tested negative in the nested PCR assay. However, another skin snip from this individual taken from the iliac crest was mf positive and was also positive in the nested PCR assay. Four individuals in this group were mf negative but showed a positive Mazzotti reaction as well as a positive result in the nested PCR assay.

DISCUSSION

We have developed a sensitive PCR assay to detect specifically *M. streptocerca* DNA in skin samples containing limited amounts of parasite material. *Mansonella streptocerca* occurs in many countries of tropical Africa and is often coendemic with *O. volvulus*, the agent of river blindness.[17] In contrast to the more pathogenic *O. volvulus*, almost nothing is known about the actual distribution of *M. streptocerca*, its prevalence and transmission dynamics. The use of the described nested PCR assay represents a great improvement in the diagnosis of this parasite and could therefore be a valuable tool for epidemiologic studies. It may also contribute to the elucidation of the relative pathogenicity of *M. streptocerca* compared with *O. volvulus*. Because severe cases of streptocerciasis in Europeans returning from endemic countries have been reported,[18] the described test is also important for travelers medicine.

In previous studies, evidence was found that the parasitologic standard skin snip diagnosis has poor sensitivity in detecting *M. streptocerca* infections. Collagenase digestion of skin biopsies revealed that two thirds of the total number of mf remain in the biopsy after overnight incubation.[2] Following chemotherapy with ivermectin, a Mazzotti reaction was observed in 37% of patients that tested parasitologically mf negative.[3] The present study is in accordance with these observations since all of the mf-positive and 73% of the mf-negative skin snips were positive in the nested PCR assay. This means that the estimated overall *M. streptocerca*-prevalence of about 80% (in adults) in the investigated village (Bubomboli)[2] is probably about 15% higher when assessed by the nested PCR assay. Therefore, it can be concluded that in this village almost every adult person is infected by this parasite.

Six days after treatment with a single dose of ivermectin, *M. streptocerca* DNA was detectable in 12 of 14 mf-negative biopsies. This again demonstrates the sensitivity of the nested PCR assay. For diagnostic purposes, the results of a Mazzotti test are recorded within two or three days following microfilaricidal treatment. Therefore, it should be possible using the nested PCR assay to prove whether a Mazzotti reaction is caused by *M. streptocerca.* Two of the 14 samples showed no mf and no detectable DNA, indicating that the clearance of mf and DNA was already complete only six days following treatment in these individuals. However, if a positive Mazzotti test result is used as indicator for the presence of *O. volvulus* infection, we suggest applying the nested *M. streptocerca* PCR assay to exclude false-positive results due to the presence of *M. steptocerca* infection.

The nested PCR assay was developed using only a small amount of parasite material present in a few clinical samples. Since the organization of the 5S rRNA gene was studied in

detail in the filaria *B. malayi,*[19] and since DNA sequence information is also available from many other filarial species of different genera,[11, 20] similar assays could be easily developed to classify larvae of other filaria species. Such PCR assays could be used to identify larvae in vectors or to identify zoonotic filarial infections in humans.

Ransohoff and others reported some variation of the 5S rDNA in the coding region in *B. malayi.*[19] We found very little intraspecific variation in the spacer region in *M. streptocerca,* but sufficient interspecific differences to design species-specific primers.

Other rDNA sequences have already been successfully analyzed to differentiate several other nematode taxa. For example, trichostrongyloid nematodes were identified by PCR amplification of their second internal transcribed spacer from the 5.8S/28S rDNA.[21] Thus, internal transcribed spacer rDNA has been analyzed to identify or differentiate nematodes, but has not been used as a target for a diagnostic PCR assay to detect nematode infections in humans. In the present study, we showed that the 5S rDNA spacer region is a suitable target sequence for a diagnostic PCR assay and that a high degree of sensitivity and specificity can be achieved.

Mansonella streptocerca can be specifically detected in skin biopsies by the amplification of the 5S rDNA spacer region using a nested PCR assay with high sensitivity. This test improves the diagnosis of the parasite and may facilitate further epidemiologic studies. The assay may help to exclude problems that arise from the confusion of *M. streptocerca* and *O. volvulus* and aid in the effort to control onchocerciasis.

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