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Spinacia oleracea L. Leaf Stomata Harboring *Cryptosporidium parvum* Oocysts: a Potential Threat to Food Safety▽†

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Cryptosporidium parvum **is a cosmopolitan microscopic protozoan parasite that causes severe diarrheal disease (cryptosporidiosis) in mammals, including humans and livestock. There is growing evidence of *Cryptosporidium* persistence in fresh produce that may result in food-borne infection, including sporadic cases as well as outbreaks. However, drinking and recreational waters are still considered the major sources of *Cryptosporidium* infection in humans, which has resulted in prioritization of studies of parasite etiology in aquatic environments, while the mechanisms of transmission and parasite persistence on edible plants remain poorly understood. Using laser scanning confocal microscopy together with fluorescein-labeled monoclonal antibodies, *C. parvum* oocysts were found to strongly adhere to spinach plants after contact with contaminated water, to infiltrate through the stomatal openings in spinach leaves, and to persist at the mesophyll level. These findings and the fact that this pathogenic parasite resists washing and disinfection raise concerns regarding food safety.**

Cryptosporidiosis is typically considered a disease transmitted by direct person-to-person and zoonotic exposure and by ingestion of drinking or recreational water contaminated with the environmentally resistant oocyst stage excreted in the feces of infected humans and animals. However, a growing shift in consumer dietary habits toward fresh and organically grown produce correlates with an increased occurrence of food-borne outbreaks of *Cryptosporidium* infection (3, 4, 10, 20, 30, 33). Irrigation waters have been suggested to be among the major routes of *Cryptosporidium* contamination of fresh produce (6, 8, 29, 32, 36). For instance, 36% of waters used to irrigate crops traditionally eaten raw in the United States and Central America tested positive for *Cryptosporidium parvum* oocysts (36). Forty-eight percent of irrigation waters examined in Mexico contained *Cryptosporidium* oocysts (6). Irrigation waters in Norway were also found to be contaminated with *C. parvum* (32). Vegetal produce can also be contaminated with oocysts during postharvest washing (14). Indeed, *Cryptosporidium* oocysts were found in wash water tanks in 16% of vegetable-packing houses in Mexico (6). Disinfection of water tanks is difficult because *Cryptosporidium* can be protected in complex bacterial biofilms colonizing both water reservoirs and distribution systems (16). Oocysts can be released with the passage of time from the biofilms, causing secondary contamination of water following an initial contamination event (1). Chemicals used to disinfect drinking and industrial water, such as chlorine and chloramine, are not effective for killing *Cryptosporidium* at concentrations typically applied (22). Moreover, some oocysts have retained infectivity even after exposure to concentrated laundry bleach for 2 h (11).

In the United States spinach is eaten raw mostly as a component of fresh salads. From 1992 to 2004 fresh spinach consumption in the United States increased by 180%, from less than 1 lb (453 g) *per capita* annually to almost 2.5 lb (1,333 g) *per capita* (25). To elucidate the mechanism of *C. parvum* persistence in fresh produce, a study was conducted using spinach plants grown either hydroponically or in soil. Laser scanning confocal microscopy (LSCM) facilitated by application of fluorescein-labeled monoclonal antibodies against *Cryptosporidium* oocysts was used to examine plants for the presence of oocysts on leaves and roots after exposure to waterborne oocysts and after washing of harvested plants.

MATERIALS AND METHODS

Purification of *C. parvum* oocysts. *C. parvum* was propagated in 2-week-old calves at the Beltsville Agricultural Center, Beltsville, MD, and oocysts were purified and quantified as described by Fayer et al. (12).

Plant growth and inoculation. Cultivar Falcon hybrid spinach seeds were obtained from a commercial source (Seminis, Oxnard, CA). Seeds were surface sterilized for 30 min with 1.0% (vol/vol) sodium hypochlorite, which was followed by five washes with sterile deionized water. To promote uniform and vigorous germination, seeds were primed in a solution containing 30% polyethylene glycol (PEG) 8000 for 72 h as recommended by Hart et al. (15). After priming, PEG was removed from the seeds by washing, and they were planted in 1-in. pots using Metro-Mix 360 soil mixture (SUN GRO, Bellevue, WA). Germination and seedling growth were carried out in a growth chamber at 25°C and a relative humidity of 50 to 60% using a photoperiod consisting of 18 h of light (600 μmol m^{-2} s^{-1}) and 6 h of darkness. Approximately 80% of the seedlings emerged from the soil within 3 to 6 days after planting. Fertilizer (Miracle-Gro, Marysville, OH) was applied every 3 days as a soil drench. Two weeks later, the most vigorous seedlings were spiked with water containing 1,000 *C. parvum* oocysts/ml. Water was sprayed perpendicular and parallel to the leaf surface using a manual spray

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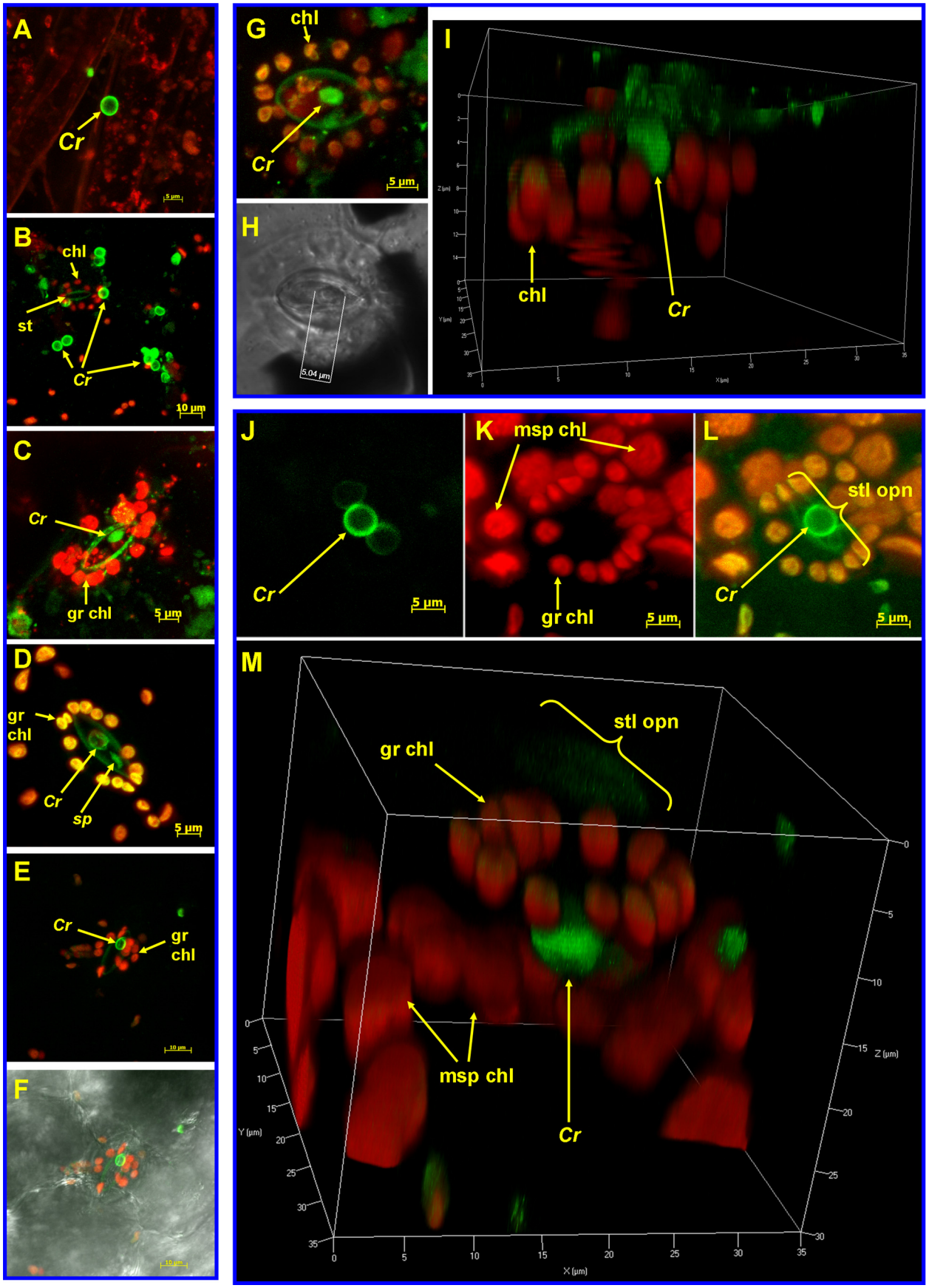

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bottle. The spraying reached or slightly exceeded the canopy spray runoff point. After contamination, plants were surface irrigated with sterile water daily, using the same spray trajectory from an identical spray bottle. Leaf samples were collected for microscopic analysis on the second, third, and fifth days after contamination.

To study the adherence of oocysts to spinach plants grown hydroponically, PEG-primed spinach seeds were placed in 20-mm-diameter petri dishes with 20 ml of 10% (vol/vol) Hoagland basal salt mixture (18) and grown with gentle agitation under the conditions described above. Seedlings with a well-developed root system and with two to three emerged leaves were transferred to petri dishes, each of which contained 20,000 *C. parvum* oocysts in 20 ml of 10% (vol/vol) Hoagland solution, and grown for two more days.

Preparation of vegetal tissue and labeling of oocysts for microscopy. Spinach roots cut from hydroponically grown plants were subjected to prolonged (up to 12 h) intensive washing (orbital shaker at 130 rpm) in 1 M glycine (pH 5.5) elution buffer (7). The same intensive washing procedure was used to wash spinach leaves from plants grown in soil. After washing, roots and leaves were rinsed with sterile deionized water and incubated with a MeriFluor (Meridian Bioscience, Cincinnati, OH) solution for 15 min. Tris-HCl (50 mM, pH 8.5) supplemented with 0.1% (vol/vol) Tween 20 and 0.5% (vol/wt) sodium dodecyl sulfate (SDS) was used to rinse unbound Merifluor antibody from the samples. Excess buffer was removed by repeatedly submerging samples in sterile deionized water. Samples were then placed on cover glasses on the bottom of petri dishes (MatTeck Corp., Ashland, MA) and immersed in a biological buffer [10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5] for microscopic examination.

Laser scanning confocal microscopy. A Zeiss 710 laser scanning confocal microscopy (LSCM) system was utilized. The images were observed using a Zeiss Axio Observer inverted microscope with 40 × 1.2 NA water immersion and 63 × 1.4 NA oil immersion Plan apochromatic objectives. Differential interference contrast (DIC) and confocal fluorescence images were acquired simultaneously. A photomultiplier tube captured the light emitted from a 488-nm argon laser with a 3.7-μm pin hole passing through an MBS 488 filter with limits set between 492 and 543 nm for detection of fluorescein and between 647 and 721 nm for detection of autofluorescence from chloroplasts. Zeiss Zen 2008 software was used to obtain the images with 512 × 512 pixel resolution, 6:1 zoom, and a *z* stack of 35 to 60 focal planes. Zeiss AxioVision version 4.8.3 with 4D software was used to construct three-dimensional images of specimens.

RESULTS

Cryptosporidium oocysts adhered to root hairs of spinach plants grown in hydroponic medium containing suspended oocysts. Prolonged (≥12-h) intensive washing of the roots in an elution buffer did not dissociate oocysts (Fig. 1A). Strong adherence of oocysts to spinach leaves was also observed after plants were sprayed with an aqueous suspension of *C. parvum* oocysts (1,000 oocysts/ml). Oocysts were detected on the surface of leaves 2, 3, and 5 days after exposure despite daily postcontamination irrigation of plants with sterile water (Fig. 1B). Furthermore, LSCM analysis revealed multiple instances of oocysts captured within stomata (Fig. 1C to F). A rarely observed free sporozoite was present in the immediate vicinity of an open oocyst (Fig. 1D). The two-channel confocal image in Fig. 1G shows another oocyst internalized within a stomate. A differential interface contrast (DIC) micrograph of the same area (Fig. 1H) clearly shows that this oocyst is localized below the level of the guard cells. A three-dimensional projection image (Fig. 1I) generated from 35 consecutive focal planes shows the spatial localization of the same oocyst within the stomate 6 μm below the leaf epidermis. Movie S1 in the supplemental material is a video file composed of 35 frames showing the progress of image acquisition (along the *z* axis within a 14-μm range) from the leaf epidermis to the underlying mesophyll. Another example of the internalization potential of the parasite is documented in Fig. 1J to M. Three *Cryptosporidium* oocysts were found deep beneath the stomate opening at the level of the mesophyll layer 15 to 25 μm below the leaf surface (Fig. 1J). The complete lack of fluorescence emitted by oocysts in the 647- to 721-nm range provides supplementary evidence that these structures do not have a vegetal origin, and they are not in a red channel image showing the mesophyll and guard cell chloroplasts (Fig. 1K). A confocal combined two-channel image shows oocysts surrounded by leaf chloroplasts (Fig. 1L). A three-dimensional projection image (Fig. 1M) and a video of 60 consecutive focal planes (a 30-μm range) of a spinach leaf (see Movie S2 in the supplemental material) demonstrate that even relatively large oocysts (diameter, 5 μm) can be easily internalized deep within the plant tissue.

DISCUSSION

Under experimental conditions, oocysts of *C. parvum*, an environmentally resistant, abundant, and ubiquitous human pathogen, strongly adhered to the roots and leaves of an edible plant and resisted removal by vigorous washing. Oocysts were also internalized within the leaves, where washing is totally ineffective. This is the first evidence of *C. parvum* internalization inside an edible leafy green vegetable and indeed is the first time that any protozoan parasite capable of infecting humans or animals has been shown to be sequestered within fresh vegetable produce. Extension of these findings to the possibility and likelihood that such parasites occur under natural conditions raises concerns regarding food safety.

Clinical trials with human volunteers have demonstrated that ingestion of as few as nine *C. parvum* oocysts is sufficient to initiate disease in immunocompetent individuals (27). Whereas exposure to *C. parvum* in healthy individuals can result in a transient infection ranging from asymptomatic to severe, in immunocompromised patients in the absence of

FIG. 1. Detection of *Cryptosporidium* oocysts on spinach plants utilizing an LSCM and fluorescein-labeled monoclonal antibodies. (A) Multichannel confocal image (MCI) of a *Cryptosporidium* oocyst (*Cr*) attached to a root hair (red fluorescence) of spinach. (B) MCI of multiple *Cryptosporidium* oocysts on the surface of a spinach leaf in the vicinity of the stomata (st). Chloroplasts (chl) fluoresce red. (C) MCI of a *Cryptosporidium* oocyst trapped within a stomatal opening on a spinach leaf. The guard cell chloroplasts (gr chl) are arranged in a circle and fluoresce red. (D) Another example of an oocyst captured between the guard cells, also showing a free *Cryptosporidium* sporozoite (*sp*). (E) MCI and (F) MCI overlaid with a DIC image of the same area showing a *Cryptosporidium* oocyst trapped within a stomate on a different spinach leaf. (G) MCI of a *Cryptosporidium* oocyst trapped within a stomatal opening on a spinach leaf. (H) DIC image of the same area showing the actual size of the oocyst. (I) Three-dimensional projection image showing spatial localization of oocysts within a stomate. (J) Single-channel (green) confocal image of 3 *Cryptosporidium* oocysts within a spinach leaf. (K) Single-channel (red) confocal image of the same area showing localization of the guard cell and mesophyll chloroplasts. The guard cell chloroplasts are arranged in a circle and range from 2 to 3 μm in diameter. Mesophyll chloroplasts (msp chl) are significantly larger (7 to 12 μm) and have irregular shapes. (L) MCI of the same area showing a *Cryptosporidium* oocyst trapped within a stomatal opening on a spinach leaf. *Cryptosporidium* oocysts are visible through the stomatal opening (stl opn). (M) Three-dimensional projection image showing internalized oocysts at the mesophyll level below the guard cells (stomate).

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efficient drug therapy (34), infection can be life threatening (13, 23). Virtually the same infectious dose was found for *Cryptosporidium hominis*, the species transmitted from human to human; the 50% infectious dose in healthy adults was 10 oocysts (7). These organisms are the two predominant species that infect humans. With oocysts that are nearly the same size, morphology, and infectivity as *C. parvum* oocysts, *C. hominis* also appears to be a likely candidate for attachment to and/or internalization by plant tissue where reclaimed water from urban areas is used for irrigation.

Most spinach and other leafy green vegetables are grown in areas where there is intensive irrigation and where contamination *via* contact with contaminated irrigation water can be a major source of *Cryptosporidium*. Furthermore, the turgidity of the guard cells increases in well-watered plants, resulting in enlargement of the stomatal aperture, which could facilitate infiltration of oocysts under the epidermal cells. Oocysts passing through the stomatal opening can be trapped in the spongy mesophyll of the leaf, where they are better protected from desiccation and sunlight, possibly extending the viability of the parasite. Currently, there are no widely used practices to reduce or eliminate *Cryptosporidium* from irrigation water (natural or artificial ponds, rivers, surface and ground waters, etc.), where oocysts can remain viable and infectious for months (21). Additionally, in the field, oocysts can come into contact with fresh produce through contaminated water used to prepare solutions of fungicides and insecticides. Even if pathogen-free water could be used for irrigation and application of agrochemicals, drop splashes, such as rainfall, can deliver large quantities of substrate particles (soil, compost, livestock manure, and animal feces) potentially containing oocysts to leaves. Most of the particles would be delivered on the abaxial side of the leaf, where stomata are more numerous (especially in dicotyledons). Thus, oocyst adherence to the leaf surface or internalization in leaf mesophyll through natural pores is possible. Fresh produce could also become contaminated during postharvest processing through contaminated equipment or food handlers (14, 30, 33).

Current microscopic methods for investigating oocyst contamination of food have technological limitations that lead to underestimation of contamination or misidentification of organisms that have no public health significance (35). According to Laberge et al. (24) and as reported by the Centers for Disease Control and Prevention (5), the annual number of cases of cryptosporidiosis is highly underreported and as a result is underestimated. An estimate of the true prevalence of salmonellosis compared with the number of reported cases suggests that the latter is about 1 to 5% of the former (17). Using this factor for cryptosporidiosis suggests a true prevalence (disease burden) for cryptosporidiosis of 165,380 to 826,900 cases, compared to the 8,269 cases reported annually to the Centers for Disease Control and Prevention (5). In Scandinavia the number of cryptosporidiosis infections is also greatly underestimated in national registers of infectious diseases, since a single registered case represents from 4,072 to 15,181 undetected or unregistered cases (19). There are several reasons for such a low number of case reports, including (i) the difficulty associated with diagnostic detection and identification of *Cryptosporidium* (30), (ii) the fact that immunocompetent persons with diarrhea rarely seek medical help (13, 23), (iii) the fact that testing for causes of diarrheal disease is not routinely conducted by most medical laboratories in the United States and Europe (26). In addition, for over one-half the reported food-borne outbreaks, the etiological agent remains unknown (9).

Even after the pathogen causing a food-borne outbreak has been identified, finding the actual produce harboring the pathogen is a significant challenge. Detection of protozoan parasites in fresh produce is especially complicated because unlike bacteria, encysted protozoans do not multiply on or within plant tissue and do not reproduce on nutrient media. Current practices for identification of pathogenic protozoans in fresh produce are based on PCR detection of parasite DNA in produce eluates or direct microscopic analysis of concentrated produce eluates and, to a much more limited extent, actual food matrices (2, 6, 31, 32). The U.S. Food and Drug Administration protocol to test fresh produce for *Cryptosporidium* sp. and *Cyclospora* contamination also relies on an immunomagnetic bead system combined with immunofluorescence microscopy and is based on the concentration of oocysts obtained from produce washes (28). Results of the present study indicate that existing elution techniques do not ensure complete recovery of oocysts from plant tissue, because oocysts remain adhered to plant surfaces and within pores. Because of the limitations of patient diagnostic tests and of methods for detection of parasites in food matrices, cases of cryptosporidiosis, particularly food-borne cases, are significantly underreported, and thus the level of the potential risk associated with the consumption of raw vegetal produce is underestimated.

In the present study a free sporozoite was observed near open oocysts in stomata of a spinach leaf (Fig. 1D). Oocysts of *Cryptosporidium* contain four sporozoites that are protected by the oocyst wall from damaging environmental conditions. When oocysts are ingested, they release these sporozoites, which initiate infection. Some oocysts also release sporozoites when the ambient temperature reaches ~37°C (13). The presence of a free sporozoite on a spinach leaf may be explained by the warming effect of laser scanning on the leaf during microscopic analysis of the specimen.

The present study demonstrated that oocysts of *C. parvum* can firmly attach to the roots and even internalize in the leaves of an edible plant that could potentially serve as a transmission vehicle for *Cryptosporidium*. Even prolonged and intensive washing of the roots and leaves in an elution buffer developed and recommended for use in analytical laboratories to test fresh produce for *Cryptosporidium* sp. and *Giardia* sp. contamination (8) was unable to dissociate all oocysts. Oocysts internalized in plant tissue have even greater protection from environmental degradation and removal. Internalized oocysts are shielded from brushing, sonication, and other physical and chemical treatments used during postharvest processing of fresh produce, making removal difficult if not impossible. Additional guidelines for food safety should take this phenomenon into account.

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