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CHARACTERISTICS AND IMPORTANCE OF THE GENUS *PROTOTHECA* IN HUMAN AND VETERINARY MEDICINE

ABSTRACT: *Prototheca* spp. are strange algae, assigned to the genus *Prototheca*, family *Chlorelaceae.* They are ubiquitous in nature, living predominantly in aqueous locales containing decomposing plant material. *Prototheca* spp. were isolated from skin scarificates, sputum and feces of humans in absence of infection, as well as in a variety of domestic and some wild animals. *Prototheca* spp. are unicellular organisms, oval or spheric in shape. They differ from bacteria and fungi in size, shape and reproductive characteristics. Of the five known species of the genus, only *P. wickerhamii* and *P. zopfii* are considered pathogenic, and they are the only known plant causative agents of human and animal infections. Over the past 25 years medical references reported more than 100 cases of human protothecoses, mostly induced by *P. wickerhamii* and rarely by *P. zopfii.* A half of the reports on human protothecoses relates to localized cutaneous infections and oleocranon bursitis. The rarest and most severe form of the infection is disseminated or systemic protothecosis, described in patients with durable course of primary disease or immune disfunction. In veterinary medicine, *Prototheca zopfii* and rarely also *P. wickerhamii* are reported as causative agents of cutaneous protothecosis in dogs and cats, systemic protothecosis in dogs and mastitis in dairy cows. Protothecal infections are diagnosed by histopathology examination or, more exactly, by isolation of the agent, although the organism cannot be distinguished from the yeasts by its cultural characteristics. Final diagnosis is made by the carbon-hydrate assimilation test. Protothecal infections are easily missed in routine practice. Pharmacological protocol for therapy of this rare infection has not been developed yet either in human or in veterinary medicine. Several antifungal agents are aplied for treatment; however, the effects are variable. Where possible, surgical excision is treatment of choice. Prognosis is promising in patients with localized infection, and healing is often achieved. Prognosis is less predictable, mostly bad, in patients with other diseases and in immunocompromized patients. Sensitivity of *Prototheca* spp. *in vitro* does not necessarily correlate with its efficacy *in vivo*.

KEY WORDS: *Prototheca* spp., Clinical signifikance, Diagnosis

In 1880, Zopf and Kühn first isolated unknown organisms from the slime flux of a linden tree. The isolates were morphologically and physiolo-

gically characterized in 1894 by Krüger, who identified them as fungi on account of their cultural similarity to yeasts (Krüger, 1894). The organisms were identified as algae in 1913 due to their ability to sporulate similarly as *Chlorella* species, and reclassified three years later by West (West, 1916) to the genus *Prototheca*, family *Chlorelaceae*. *Prototheca* is an achlorophyllous mutant of the green algae with nonfunctional chloroplast. Achlorophyllous mutants of *Chlorellae* have been successfully produced in the laboratory; although the pathway of their genesis in natural environment still remains unclear (Huss and Sogin, 1990). Until now, five species have been generally recognized within the genus: *P. wickerhamii, P. zopfii, P. moriformis, P. ulmea* and *P. stagnora*.

DISTRIBUTION

Prototheca spp. is ubiquitous in nature and widely distributed all over the world. The organism had first been isolated from the slime flux of trees and over the following decades from a wide variety of sources including sewage, soil, plants and many fresh and salt water sources, even from water-supply systems (Anderson and Walker, 1998; Pore et al., 1983, Walsh et al., 1998). The organism is particularly predominant in aqueous locales containing decomposing plant material. *Prototheca* spp. were isolated from skin scarificates, sputum and feces of humans in absence of infection (Sonck and Koch, 1971), as well as in a variety of domestic and some wild animals. Occurrence of the organism in the feces of cattle, pig, horses and sheep is associated with the use of contaminated feed.

Life cycle and morphological traits

Prototheca spp. are monocellular organisms, oval or spherical in shape, 7—16 μm in diameter (Anderson and Walker, 1998; Di Persio, 2001). They differ from bacteria and yeasts in size, shape and reproductive characteristics. Contrary to yeasts and bacteria they do not have glucosamine in their cell wall and do not contain muraminic acid, respectively (Di Persio, 2001). The cell wall of *Protothecae* consists of outer (thinner) and inner (thicker) envelope, while all *Chlorella* species (except for *Ch. prototecoides*) are characterized by a three-layer cell wall (Sudman, 1974). The three species commonly isolated from the natural environment are considered non-pathogenic, i. e., *P. stagnora, P. ulmea* and *P. moriformis*. These species are able to produce capsule (similarly to *Cryptococcus neoformans*); however, this feature was not observed in any of clinical isolates of *P. wickerhamii* and *P. zopfii* (Di Persio, 2001). The reproduction is asexual, by internal septation and endospore formation. The sporangia contain 2—16 or more daughter-cells (sporangiospores), which, following the characteristic cell-wall breakage, further develop the endosporulating cells (Pore, 1998).

Prototheca spp. as causative agent of infection

Of the five known species of the genus, only *P. wickerhamii* and *P. zopfii* are considered pathogenic, yet their pathogenic potential is low. Until now, they are the only known plant causative agent of human and animal infections (Roesler et al., 2003).

Human protothecoses

The first case of human protothecosis was described by Davies et al. in 1964, manifested as localized skin lesions in a farmer in Sierra Leone, while the first systemic protothecosis was described by Cox et al. in 1974. Over the past 25 years, medical references reported on more than 100 cases of human protothecoses, mostly induced by *P. wickerhamii* and rarely by *P. zopfii* (Krcmery, 2000; Monopoli et al., 1995; Wirth, 1999). Though unfrequent, protothecal infections are reported worldwide: in Europe, Asia, Africa and Central America. In our country, the first isolation of *Prototheca* spp. from clinical material was reported on April 5, 2005 by the Serbian Microbiology Association, Section of Vojvodina (Suvajdžić, 2005). Infections occur in all age categories, but extremely rarely in the pediatric population.

Described as environment-borne agent, *Prototheca* spp. enter the body via the traumatic skin lesion and mucous membranes, on subsequent exposure to contaminated water. Human-human transmission route is excluded. The incubation period has not been precisely defined yet; however, anamnestic data on post-traumatic infection suggest a 2-week incubation period.

Some 50% of all reports on human protothecoses relate to localized cutaneous infections, slowly-developing single or multiple lesions that do not heal spontaneously. They are described as diffuse erythema, papulae, vesico-pustules, ulcerations, nodous, eczematous or herpetiform changes located mostly on extremities. Pain and swelling of soft tissues may occur, as well as various quantities of serous-sanguineous liquid. Regional lymph nodes are rarely affected. Failure of prompt diagnosis of cutanous protothecosis may result in the development of chronic destructive lesions even in immunocompetent individuals. The second most frequent form of protothecosis is olecranon bursitis, which develops as a consequence of traumatic implantation of the agent. It is manifested as induration of the bursa, associated by swelling and moderate erythema (Galan et al., 1997). The rarest and most severe form of the infection is disseminated or systemic protothecosis, described in patients with durable course of primary disease or immune dysfunction (*diabetes mellitus*, malignancy and chemotherapy, renal transplantation, systemic *lupus erythematosus*, corticosteroid therapy and HIV infection) (Kunova et al., 1996).

Animal protothecoses

In veterinary medicine, *Prototheca zopfii* and rarely also *P. wickerhamii* are reported as causative agents of cutaneous protothecosis in dogs and cats,

systemic protothecosis in dogs and mastitis in dairy cows (Gonzales, 1996, Monopoli et al., 1995).

Dogs and cats

Cutaneous protothecosis induced by *P. wickerhamii* is the only disease manifestation described in cats (Dillberger et al., 1988). In both cats and dogs, the cutaneous form results from injury infection and is manifested by the occurrence of ulcerative lesions, scabs and pyogranulomatous dermatitis in limbs, trunk and mucous surfaces. Hyperkeratosis may develop too, and frequent complications are due to secondary bacterial infections. Pathohistological examination of skin bioptates reveals abundant protothecal organisms within the cytoplasm of phagocytic cells (Ginel et al., 1997).

In systemic protothecosis in dogs it is most likely that algae enter the body by ingestion, passing the intestinal mucosa (infection portal) and disseminating to the entire body via the hematogenic and lymphogenic routes (Hollingsworth, 2000). Systemic prorothecosis in dogs is mostly induced by *P. zopfii*, rarely by *P. wickehamii*, and disease symptoms depend on the organs and organ-systems affected, as well as on the severity of the lesions. Unremarkable and unspecific symptoms mostly result in delayed diagnosis, enabling the agent to spread over the entire body. Even in systemic infections only cutaneous manifestations, symptoms of gastro-intestinal disorders and eye infection were clinically manifested (Hollingsworth, 2000; Ginel et al., 1997).

Numerous reports have indicated gastrointestinal disorders as most frequent clinical signs of disseminated protothecosis. Migaki et al. (1982) described hemorrhagic colitis in dogs induced by *P. zopfii.* The colon is the most severely affected site, but lesions are visible over the entire intestine. Clinical manifestation of the disease includes vomiting, tenesmus and intermittent diarrhea (blood and slime in the feces). Colonoscopy reveals diffuse hyperemia, hemorrhages and ulcerations, as well as multiple granuloma of the mucosa, which, in the further course of the disease, results in intestinal stricture and obstipation.

The infection can spread to the central nervous system, cardio-vascular system and urinary tract, liver, skeletal muscles, lymph nodes, thyroid gland, pancreas, peritoneum and diaphragm (Hollingsworth, 2000). Infection of the eye is common manifestation of systemic protothecosis in dogs. It results in severe damages and blindness that are due to development of glaucoma and retinal ablation. *Protothecae* are identified by microscopy examination of *humor vitreus* (Schultze et al., 1998). Manifest signs of disease in CNS (cervical pain, depression, ataxia, pareses) are rare. The affected organs exhibit typical reaction to protothecal infection, i.e., formation of granulomes 0.5—2 mm in diameter with the cell infiltrate composed of cell plasma, macrophages and neutrophiles.

Bovine mastitis

Prototheca was first linked to mastitis by Lerch in 1952. Mastitis in dairy cows is mostly induced by *P. zopfii*, sometimes by *P. wickerhamii* (Gonzales, 1996; Janosi et al., 1998). *P. zopfii* mastitis is a disease of highly productive cows in the machine-milking systems. Although it mostly occurs sporadically, endemic events are also reported (Janosi et al., 2000). *Prototheca spp*. as well as species of *Staphylococcus, Streptococcus, Arcanobacterium pyogenes* and *Mycoplasma* may be of great significance for the herd (Boboš and Vidić, 2003), especially if infection is not timely diagnosed. Similarly to some bacterial species such as *Arcanobacterium pyogenes, Pasteurella multocida* or *Corynebacterium ulcerans*, *Prototheca* is rarely identified as the mastitis agent (Suvajdžić et al., 2001b; Suvajdžić et al., 2003; Suvajdžić et al., 2001). The wide distribution of these algae in the environment and their occurrence and isolation at the farms showing no sign of protothecal mastitis indicate the crucial role of predisposing factors, such as poor milking hygiene or prolonged antimicrobial therapy of mastitis of other etiologies (Anderson and Walker, 1998; Schlendstedt et al., 1997; Tenhagen et al., 1999). Outbreaks of seasonal protothecal mastitis have been reported during warm and humid periods of the year that promote the propagation of these organisms in their natural habitat (Costa et al., 1996).

Infection commences at the papillae of mammary glands in all lactation phases, including dry period (www.Uvex.edu/miklquality/prototheca). In the first weeks of lactation an increased susceptibility to infection was observed, which is due to the pronounced energetic misbalance (Costa et al., 1997). The infective dose is not yet defined. In experimental conditions, infusion of 40—480 CFU *P. zopfii* results in mastitis in 100% cases. Five days after the experimental infection the alga can be isolated from milk in a yield 20 000—50 000 CFU/ml (McDonald et al., 1984a). Anticipated infective doses for natural infection are high in comparison with other mastitis agents. This quantity is provided through permanent contact of teeth ends with the primary infection sources such as soil, plants, water sources and feces. A common infection source is improper sanitation before the mastitis treatment, since the organism may be introduced via the infusion material, similar as *Nocardia* species. Furthermore, cow-to-cow infection during milking is also possible (Anderson and Walker, 1998; Dion, 1979). *Prototheca* spp. have been recovered from the rubber parts of milking equipment and they showed height resistance to routine disinfection with chlorine solutions (Anderson and Walker, 1998; Costa et al., 1997). Despite the observed mixed infections with *Staphylococcus aureus* and *Streptococcus* spp., synergy of *P. zopfii* and other bacteria pathogenic for bovine udder was not confirmed (Schlensted et al., 1997).

Protothecal infections result in chronic manifest subclinical and clinical mastitis without signs of systemic infection (fever, depression) (Tenhagen et al., 1999). Compared with mastitis forms of other etiologies, the inflammatory process is milder during the acute phase. Besides the moderate course

of the disease and pain, decrease in milk production is observed, associated with somewhat altered, watery secretion containing clots. The usual antimicrobial therapy brings no improvement. Somatic cell count is mostly higher than 1 million/ml; however, individual values may range between 6 and 9 millions/ml (J a n o s i et al., 1998), sometimes even exceeding 20 millions/ml (M a l i n o w s k i et al., 2002). In that respect, even a limited infection may affect the milk quality within herd. Pursuant to our Regulations on Hygienic Safety of Milk, the threshold value for somatic cell count in hygienically safe milk is 500,000/ml, while regulations of EU countries do not allow values higher then 300,000/ml (B o b o š et al., 1997). If protothecal infection, especially subclinical one, is present in a herd, the somatic cell count in bulk milk samples may be the indicator of the suspect agent.

Since there is neither effective therapy nor spontaneous cure, protothecal infections of the mammary gland become chronic and persist through several lactating periods. Algae are limited to the mammary gland and regional lymph nodes exhibiting granulomatous inflammation. Histological lesions are characterized by massive aggregation of macrophages, plasma cells and lymphocytes (B o b o š and V i d i ć, 2003). Sporangia and sporangiospores are inside the macrophage and neutrophiles in the alveolar lumen and interstitium, suggesting that intracellular proliferation is responsible for inability to overcome the infection (J a n o s i et al., 1998). A pathohistological examination of udder tissue in chronic disease course has revealed the progressive interstitial mastitis with consequent alveolar atrophy (J a n o s i et al., 2001). Such changes of the mammary gland result in a progressive drop in milk production (C o s t a et al., 1997; M c D o n a l d et al., 1984b; T e n h a g e n et al., 1999).

Attempts to locate sites containing *Protothecae* in dairy-cow housings have not proved to be successful, as examination of highly-contaminated samples from the environment does not support such measure (www.Uvex.edu/mikl-quality/prototheca). Cows should be kept out of humid areas, particularly these containing manure and decomposing plant material in order to prevent the papillae from exposure to the infection sources (C o s t a et al., 1996). Proper draining system will lower the number of microorganisms in the environment. It is of paramount importance to prevent contact of the teeth ends with feces and moist manure 25—30 minutes after milking because of the relaxed papilla sphincter (J a n o s i et al., 1998). If *Prototheca* is isolated from milk samples, it is recommended to examine all cows in the herd (H o d g e s et al., 1985). Similar to the *Mycoplasma bovis* and *Staphylococcus aureus*, *Protothecae* are intermittently shed in the milk (G o n z a l e s, 1996). As they may be transferred from cow to cow during milking, it is necessary to separate the infected animals and milk them last until final exclusion from the herd (A n d e r s o n and W a l k e r, 1998). Sporadic occurrence of mastitis justifies the culling of infected animals as a measure for prevention of further spread of the infection.

A failure to promptly diagnose the infection and to apply measures for prevention of its spreading inevitably results in the culling of infected animals and decreased milk production and quality, which may cause significant economic losses at dairy farms. The program for mastitis control must include also the *Prototheca* algae (S c h l e n s t e d et al., 1997). Pursuant to legislative regulations in our country, only the exclusion of *Staphylococcus aureus* and

Streptococcus agalactiae is obligatory. As identification of protothecal infections is not a part of routine microbiological examination in veterinary medicine, reports on their occurrence are extremely sporadical (M i l a n o v, 2004; M i l a n o v, 2005). A more complex diagnostic approach including a longer incubation period and use of restrictive media should become a regular practice in diagnosing clinical and subclinical mastitis (S u v a j d ž i ć et al., 2001a).

Milk and dairy products contaminated with *P. zopfii* are potential sources of human infection. An examination of the susceptibility of 40 strains of *P. zopfii* isolated from cow milk to different heat-treatment regimens (72—75°C/15 sec; 72—75°C/20 sec and 62—65°C/30 minutes) revealed resistance in at least one of the tests in 34 of the examined strains (M e l v i l l e et al., 1999).

Diagnosis

Protothecoses are diagnosed by histopathological examination or, more exactly, by isolation of disease agent.

Histopathological examination

Prototheca spp. are haematoxilinophyllic, hence, staining of tissue section by haematoxilin-eosin is not the most appropriate method for their identification. Visualization of the organism is achieved by *Gomori-methenamine silver* or *periodic acid-schift* (PAS) staining techniques when "morulae" are visible, mostly bigger than the ones observed *in vitro* (10—30 μm). Empty sporangia resemble fungi. The sporangia of *Prototheca* spp. are smaller and contain fewer spores in comparison with *Coccidioides immitis* and *Rhinosporidium seeberi.* The organism differs from *Blastomyces dermatitidis* and *Cryptococcus neoformans* by the size, internal structure and lack of budding (D i P e r s i o, 2001). For identification of *Protothecae* in tissue specimens, the immunofluorescency technique is applied (S u d m a n and K a p l a n, 1973).

Isolation

Prototheca spp. can be recovered (*intra vitam*) from samples of skin scarificates (cutaneous protothecosis), joint punctuate (bursitis olecranona), feces (hemorrhagic diarrhea in dogs), urine (urinary infections in dogs) and cow milk (protothecal mastitis). Material is inoculated on blood agar and Sabouraud dextrose agar using standard laboratory techniques. Plates are incubated for 48—72 h at 25—37°C (M i l a n o v et al., 2003q).

Cultural and biochemical features

Prototheca spp. grows easily on most standard laboratory nutritive media such as blood agar and Sabouraud-dextrose agar. For isolation from contami-

nated samples, application of selective media containing inhibitors of normal microbial flora is recommended. The growth of *Prototheca* spp. is inhibited by cycloheximide, yet not by hloramphenicol. A special *Prototheca* medium contains folate for inhibition of bacterial growth and 5-florocytosine for suppression of yeast growth. After incubation for 24—72 h at 25—37°C, visible *P. wickerhamii* colonies are formed which are smooth, white and yeast-like (Di Persio, 2001). Colonies of *P. zopfii* on Sabouraud-dextrose agar are clearly formed in primary culture after 48—72 hours of incubation. They are mostly large, irregularly margined, with granular surface and a compact central protrusion (Janosi et al., 1998; Milanov et al., 2004). Colonies grown on blood agar are mostly very small and pale gray (Milanov et al., 2004). If the isolation medium does not contain growth inhibitors, *Prototheca* colonies may be overgrown (covered) by bacteria after prolonged incubation (Di Persio, 2001). Since it is slow-growing, the organism may easily be missed in routine practice if incubation is terminated after 24 h (Tenhagen et al., 1999). The colonies are of creamy consistency, readily suspended in saline solution when making microscopy slides. Microscopy is indispensable to distinguish *Prototheca* spp. from yeasts, since differentiation is not possible on the basis of their cultural features.

Characteristic microscopic appearance of *Prototheca* spp. is best observed by examining native preparations using phase-contrast microscopy, when formations described as "morula" or "mulberry" is visible. *Prototheca* spp. is easily methyl-blue and Gramm-stained, but heat-fixation may induce morphological impairment. In Gram-stained preparations, positively stained spores and Gram-negative empty sporangia are visible (34). Sporangia of *P. wickerhamii* are 7—13 μm, of *P. zopfii* 14—16 μm in diameter. Final diagnosis is made by the carbon-hydrate assimilation test (API 20C Bio Merieux; VITEK® Yeast Biochemical Card; RapID Yeast Plus System-Remel). All *Protothecae* use glucose as the carbon source. Trechalose assimilation is the key parameter for differentiation between the two pathogenic strains. According to phenotype and genetic criteria, including growth- and biochemical characteristics and serotyping, various isolates of *P. zopfii* are classified into three biotypes. Biotype II, which ferments glucose and glycerol but not galactose, is mostly isolated in bovine mastitis and human enteropathies (Roesler et al., 2003).

Therapy and prognosis

Pharmacological protocol for therapy of this rare infection has not been developed yet either in human or in veterinary medicine. Several antifungal agents are applied for treatment; however, the observed effects are variable. In human medicine, Amphotericin B, a product of *Streptomyces nodosus* (Suvajdžić, 2004), is used for therapy of disseminated protothecosis. This is a funghistatic or fungicide that inhibits ergosterole synthesis in the cell membrane of *Prototheca* spp. The drug has proven to be nephrotoxic, and confirmed oversusceptibility is contraindication to its use. Administration of immidazoles, such as itracosanole, ketoconasole, fluconasole and clorimazole, may show so-

me improvement; however, their efficacy is not yet confirmed due to variable clinical response. Where possible, surgical excision is the treatment of choice. Prognosis is promising in patients with localized infection, and healing is often achieved. Prognosis is less predictable, mostly bad, in patients with other diseases and in immunocompromised patients.

Therapy of systemic protothecosis in dogs may include amphotericin B, tetracyclin, ketoconasole, itraconasole, fluconasole and chorimazole; however, outcomes of such therapies are not yet established. Cutaneous protothecosis caused by *P. wickerhamii* requires both medicamentous treatment and surgical excision.

In vitro isolates of *P. zopfii* from milk exhibited sensitivity to amphotericin B, nystatin, polymyxin B, gentamicin and neomycin (McDonald et al., 1984b; Malinowski et al., 2002). Effective therapy of protothecal mastitis has not yet been developed and the only measure to control the spread of infection in the herd is exclusion of infected animals.

Sensitivity of *Prototheca* spp. *in vitro* does not necessarily correlate with its efficacy *in vivo*.

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ОПШТЕ КАРАКТЕРИСТИКЕ И ЗНАЧАЈ АЛГИ ГЕНУСА *PROTOTHECA* У ХУМАНОЈ И ВЕТЕРИНАРСКОЈ МЕДИЦИНИ

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Резиме

Prototheca spp. су релативно непознате алге, сврстане у генус *Prototheca*, фамилије *Chlorelaceae*. Убиквитарне су природи и њихова распрострањеност је углавном везана за влажна подручја која садрже биљну материју у распадању, али су изоловане и из кожних скарификата, спутума и фецеса људи, као и из

низа домаћих и неких врста дивљих животиња. *Prototheca* spp. су једноћелијски организми овалног или сферичног облика који се од бактерија и гљива разликују по величини, облику и начину репродукције. Од пет познатих врста овог рода, само се *P. wickerhamii* и *P. zopfii* сматрају патогенима и, досада, оне су једини познати биљни узрочни агенс инфекција људи и животиња. У последњих 25 година у медицинској литератури описано је више од 100 случајева хуманих прототекоза, углавном изазваних алгама *P. wickerhamii*, а ређе *P. zopfii*. Око половина свих извештаја хуманих прототекоза односи се на локализоване кожне инфекције и бурситис олекранона. Најређа и најтежа форма инфекције је дисеминирана или системска прототекоза, описана код пацијената са другим основним обољењем или имунолошком дисфункцијом. У ветеринарској медицини *Prototheca zopfii* а ређе и *P. wickerhamii* наводе се као узрочници кутане прототекозе паса и мачака, системске прототекозе паса и маститиса млечних крава. Прототекалне инфекције се могу дијагностиковати патохистолошким прегледом или егзактније, изолацијом узрочника, мада се од квасница не могу разликовати само на основу културелних особина. Дефинитивна дијагноза поставља се тестом угљено-хидратне асимилације. Проблем код ових инфекција је што се на њих ретко посумња и нису предмет рутинске лабораторијске праксе. Ни у хуманој ни у ветеринарској медицини нема дефинисаног фармаколошког протокола у терапији. Неколико антифунгалних агенаса користи се у терапији са различитим ефектом. Тамо где је могуће, с обзиром на локализацију, третман избора је хируршка ексцизија. Код пацијената са локализованом инфекцијом прогноза је добра и обично се постиже излечење. Прогноза код пацијената са другим обољењима и имуносупресијом мање је предвидива и може бити лоша. Осетљивост *Prototheca* spp. на антифунгалне агенсе *in vitro* не мора бити у корелацији са *in vivo* ефикасношћу.