Prototheca zopfii genotype II induces mitochondrial apoptosis in models of bovine mastitis

Muhammad Shahid1, Eduardo R. Cobo2, Liben Chen3, Paloma A. Cavalcante2, Herman W. Barkema2, Jian Gao1, Siyu Xu1, Yang Liu1, Cameron G. Knight4, John P. Kastelic2 & Bo Han1*

Prototheca zopfii is an alga increasingly isolated from bovine mastitis. Of the two genotypes of *P. zopfii* (genotype I and II (GT-I and -II)), *P. zopfii* GT-II is the genotype associated with acute mastitis and decreased milk production, although its pathogenesis is not well known. The objective was to determine inflammatory and apoptotic roles of *P. zopfii* GT-II in cultured mammary epithelial cells (from cattle and mice) and murine macrophages and using a murine model of mastitis. *Prototheca zopfii* GT-II (but not GT-I) invaded bovine and murine mammary epithelial cells (MECs) and induced apoptosis, as determined by the terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling assay. This *P. zopfii* GT-II driven apoptosis corresponded to mitochondrial pathways; mitochondrial transmembrane resistance ($\Delta \Psi_m$) was altered and modulation of mitochondrion-mediated apoptosis regulating genes changed (increased transcriptional Bax, cytochrome-c and Apaf-1 and downregulated Bcl-2), whereas caspase-9 and -3 expression increased. Apoptotic effects by *P. zopfii* GT-II were more pronounced in macrophages compared to MECs. In a murine mammary infection model, *P. zopfii* GT-II replicated in the mammary gland and caused severe inflammation with infiltration of macrophages and neutrophils and upregulation of pro-inflammatory genes (*TNF-\alpha*, *IL-1\beta* and *Cxcl-1*) and also apoptosis of epithelial cells. Thus, we concluded *P. zopfii* GT-II is a mastitis-causing pathogen that triggers severe inflammation and also mitochondrial apoptosis.

Bovine mastitis (inflammation of the udder), caused by infection with pathogenic microorganisms and destruction of milk-synthesizing tissues1, reduces milk production and quality and is an important financial threat to the dairy industry2. *Prototheca zopfii*, a chlorophyllous alga (family *Chlorellaceae*) unable to synthesize chlorophyll and with heterotrophic modes of nutrition3,4, is a major cause of mastitis in dairy cows5,6. Bovine protothecal mastitis can be clinical or subclinical. In clinical cases, symptoms include fever (up to 40°C), pain, mammary edema, anorexia and reluctance to move7. Subclinical protothecal mastitis is associated with increased number of leukocytes in the udder and milk and can be manifested by slight pain along with loss of appetite7. Bovine protothecal mastitis decreases milk production and elevates somatic cell count in milk, especially macrophages, often resulting in culling7. Reported bovine *Prototheca zopfii* mastitis occurrence ranges from 7.5 to 16.3%8,9; however, these reports are predominantly from outbreaks. Although a large proportion (up to 81%) of dairy herds are infected, this pathogen affects a limited proportion of cows (<10%)10,11. Cows are often infected intramammarily with *P. zopfii* following teat trauma during mechanical milking12 and contamination of the teat orifice with damp organic material12,13. Single *Prototheca zopfii* endospores or sporangiospores contact mammary gland epithelial cells, which are first responders, sensing their presence and initiating an inflammatory immune response. After breaching epithelial defenses, *Prototheca zopfii* may also invade macrophages of the mammary gland alveolar lumen and interstitium14, making *Prototheca zopfii* less accessible to antibiotics and diagnostic methods15.

Two genotypes of *Prototheca zopfii*, genotype I (GT-I) and genotype II (GT-II) have been isolated from bovine milk and identified16. Genotype I is predominantly isolated from environmental samples, whereas GT-II is isolated from

1Department of Clinical Veterinary Medicine, College of Veterinary Medicine, China Agricultural University, Beijing, 100193, P.R. China. 2Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, T2N 4N1, Canada. 3Whiting School of Engineering, Johns Hopkins University, Baltimore, MD, 21218, USA. 4Department of Veterinary Clinical and Diagnostic Sciences, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, T2N 4N1, Canada. *email: hanbo@cau.edu.cn
milk samples and has been reported as the causative pathogen of bovine mastitis17–19. In the latest study, the two types were named P. ciferrii and P. bovis, separately20. *Prototheca zopfii* GT-II induced oxidative stress and apoptotic death in cultured bovine mammary epithelial cells (bMECs). *Prototheca zopfii* GT-II is more pathogenic than *P. zopfii* GT-I, commonly isolated as an environmental pathogen21–22. Most recent another study reported that mammary gland infected with *P. zopfii* GT-I had no clinical signs23, but pathogenesis of protothecal mastitis due to *P. zopfii* GT-II remains elusive. Thus, we aim to determine inflammatory and apoptotic roles of *Prototheca zopfii* GT-II in cultured mammary epithelial cells (from cattle and mice) and murine macrophages and using a murine model of mastitis.

Materials and Methods

**Statement of ethics.** The current study was conducted in accordance with ethical guidelines and regulations regarding laboratory animal care and use, as described in the “Guide to the Care and Use of Experimental Animals” from the Canadian Council on Animal Care (https://www.ccac.ca/Documents/Standards/Guidelines/Experimental_Animals_Vol1.pdf). Animal use was reviewed and approved by the Animal Care Committee of the University of Calgary, Calgary, AB, Canada (protocol number AC16–0061).

**Prototheca zopfii culture.** *Prototheca zopfii* GT-II isolates were collected from milk samples of dairy cows with clinical mastitis, whereas *P. zopfii* GT-I isolates were predominantly cultured from environmental samples in China, and cultured and stored at College of Veterinary Medicine, China Agricultural University, Beijing, China25. *P. zopfii* GT-I and II were isolated from a total of 163 *P. zopfii* isolates collected from mastitic milk and environmental samples18. In this study, *P. zopfii* GT-II was only isolated from mastitic milk, whereas GT-I was recovered from environmental samples (feed, feces, water and teat cups). Both genotypes were characterized by their cellular fatty acid pattern and 18S rDNA sequences. *P. zopfii* GT-II had increased amounts of eicosadienoic acid (C20:2) compared to GT-I. Whereas both *P. zopfii* GT-I and II had high sequence similarity (99.4%), GT-II (AY940456) differed in some nucleotides from GT-I (AY973040)18. All bovine mastitis milk *P. zopfii* strains were further identified by genotype-specific PCR and restriction fragment length polymorphism analysis16,24. In our previous study, the 450 bp fragment internal amplification control was detected using Proto18–4f (GACATGGCGGAGGATTGACAGA) and Proto18–4r (AGGCACACCATCGTGAGGA) sequences. The GT-I strain was identified by Proto18–4f (GACATGGCGGAGGATTGACAGA) and Proto18–1r (GCCAAGGCCCCCGGAAAG) primers. GT-II specific amplicon (165 bp) was detected with primers Proto18–4f (GACATGGCGGAGGATTGACAGA) and Proto18–1f (GTCGCGCGGCGAAAGC)18. The *P. zopfii* genotype was further confirmed by restriction fragment length polymorphism (RFLP) analysis targeting the cytb gene fragment (599–668 bp). For this, a PCR mix (25 μL) containing cytb-F1 (5′ GyGTwGACAAyATTATGAGAG-3′) and cytb-R2 (5′-wACCATAAAnRACCATTCoGG-3′) primers (10 μM each primer), DNA template (1 μL), and 2x EasyTaq PCR supermix (TransGen Biotech, AS111–11; 12.5 μL) was amplified under specific conditions (2 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 50 °C, and 30 sec at 72 °C, with final extension of 5 min at 72 °C). The PCR products depicted a 644 base pair (bp) product compatible with *P. zopfii* as visualized by agarose gel electrophoresis (1%, wt/vol) and stained with ethidium bromide. The amplified cytb gene products (644-bp) were digested by Rsal and Tail digesting enzymes (FastDigest Enzymes, Thermo Fisher Scientific). The total mixture (30 μL) containing 10X restriction enzyme buffer (3 μL), PCR product (10 μL), enzymes (1.5 μL each), and PCR water (15 μL) was digested by Rsal (5 min at 37 °C) followed by Tail (5 min at 65 °C). The restriction products visualized on 3% agarose gels, stained with ethidium bromide, and exposed to UV light showed DNA fragments of 200 and 450 bp after Rsal/Tail digestion, compatible with product (10)

Mouse protothecal mastitis model. C57BL/6 lactating female mice (6–8 wk old; 10–14 g) were housed in specific pathogen-free facilities at the University of Calgary with ad libitum access to food and water. Mice were inoculated intramammary with either *P. zopfii* GT-II (50 μL containing 1 × 10^7 CFU/mL) or an equal volume of phosphate buffered saline (PBS) (control) in the left fourth and right fourth (L4 and R4) mammary glands. Mice were euthanized 4 d post inoculation (dpi) to collect mammary tissue samples. Tissues were mixed into TRIzol (Invitrogen, Carlsbad, CA, USA) and later homogenized for quantitative PCR (qPCR) or fixed in 10% formalin solution, embedded in paraffin wax, sectioned with a microtome (5 μm) and stained with hematoxylin and eosin (H&E; Sigma, USA) for histological examination25 and with Periodic Acid–Schiff (PAS; Sigma, USA) and Grocott–Gomori’s methenamine silver stain (GMS) as a screen for fungal organisms.

Identification of macrophages and neutrophils in murine mammary gland. Fixed murine mammary gland tissue sections were deparaffinized, dehydrated and permeabilized with PBS/Triton X-100 (0.25%, v/v) (PBS-T) buffer containing 1% donkey serum (Cat # 017–000–121) at room temperature for 10 min. Slides were blocked with PBS-T containing 10% (v/v) donkey serum and 1% (v/v) bovine serum albumin (BSA) (Sigma, USA) for 120 min at room temperature. After washing with PBS, sections were incubated with primary antibodies against mouse F4/80 (macrophages) (Cat # 4316835, BD Pharmingen™, US) and Ly-6G (neutrophils) antigens (Cat# 127609, Biolegend, US) (1:1,000 in PBS-T plus 1% BSA) for 16 h at 4 °C. Following washing with PBS-T, slides were incubated with secondary antibodies (488-conjugated AffiniPure Goat anti-Rat IgG, Cat# 135205, Jackson Immune Research, UK) (1:1,000 in PBS-T plus 1% BSA) at room temperature for 60 min and washed again with PBS-T and then incubated with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen) at room temperature for 20 min. Slides were examined with an immunofluorescence microscope (ZEISS Axio Imager M2, Carl Zeiss AG, Jena, Thuringia, Germany).
were assessed using the 2−ΔΔCT method and results presented as mean fold change of target mRNA levels in three independent experiments. Values of target mRNA were corrected relative to the normalizer combined annealing/extension for 10 s at 60 °C (total of 40 cycles). All treatments were examined in duplicate in each specific primer, in a 10 μL of cDNA, 1X SsoAdvanced Universal SYBR Green Supermix (BioRad) and 0.5 μM of each primer. Amplification was done by actin inhibition (cytochalasin D; C8273, Sigma, USA; 1 h) before inoculation. Further confirmation of phagocytic activity of macrophages was done by actin inhibition (cytochalasin D; C8273, Sigma, USA; 1 h) before inoculation.

Mitochondrial damage assay. After infection with P. zopfii, GT-I and -II, bovine MECs were collected to assess changes in mitochondrial membrane potential (ΔΨm), as determined by JC-1 (Cat# M8650, Solarbio, Beijing, China) and processed for TEM.

Transcriptional gene expression of inflammatory and apoptotic genes. Total RNA was extracted from bMECs, mMECs and murine macrophages with TRIzol reagent (Invitrogen) and converted to cDNA (RevertAid First Strand cDNA synthesis kit, Thermo Scientific). Quality of resulting RNA and cDNA were evaluated by the absorbance ratio (A260/A280 ratio) (NanoVue Spectrophotometer, GE Healthcare Bio-Sciences, Little Chalfont, Buckinghamshire, UK), which was corrected to be ~1.8–2.0 for an individual sample. Amplification of mRNA genes for TNF-α, IL-1β, IL-8/Cxcl-1, Bcl-2, Bax, Apaf-1, cytochrome-c, caspase-9 and caspase-3 was done using a CFX-96 real-time PCR system (BioRad, Hercules, CA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), penicillin (100 U/mL; Thermo Fisher Scientific) and streptomycin (100 U/mL; Thermo Fisher Scientific) in cell culture plates (Corning Inc., Corning, NY, USA). The mMECs were cultured in RPMI (Thermo Fisher Scientific) medium along with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), penicillin (100 U/mL; HyClone®, USA) and streptomycin (100 U/mL; Thermo Fisher Scientific). For experimental challenges, bMECs and macrophages (bovine and murine) were challenged with P. zopfii GT-I and GT-II suspended in DMEM/F12 to 5 × 10^5 and 1 × 10^6 CFU/mL, respectively, for up to 24 h at 37°C with 5% CO2.

Transfection electron microscopy (TEM). Bovine MECs infected with P. zopfii GT-I and -II were washed with PBS (pH 7.2), fixed with 2% glutaraldehyde and 1% paraformaldehyde (pH 7.2; Sinopharm Chemical Reagent Co., Shanghai, China) and processed for TEM.

Epithelial cell and macrophage culture. A bMEC line isolated from a cow (MAC-T) (Shanghai Jingma Biological Technology Co., Ltd. China), murine macrophages derived from mouse BALB/c monocytes ([774, provided by Dr. Eduardo R. Cobo, University of Calgary] and a murine mammary epithelial cell line (mMECs: HC11, provided by Dr. Eduardo R. Cobo, University of Calgary) were used. The bMECs and murine macrophages were cultured in HyClone™ DMEM/F12 medium (Thermo Fisher Scientific, South Logan, NH, USA) along with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), penicillin (100 U/mL; Thermo Fisher Scientific) and streptomycin (100 U/mL; Thermo Fisher Scientific) in cell culture plates (Corning Inc., Corning, NY, USA). The mMECs were cultured in RPMI (Thermo Fisher Scientific) medium along with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), penicillin (100 U/mL; HyClone®, USA) and streptomycin (100 U/mL; Thermo Fisher Scientific). For experimental challenges, bMECs and macrophages (bovine and murine) were challenged with P. zopfii GT-I and GT-II suspended in DMEM/F12 to 5 × 10^5 and 1 × 10^6 CFU/mL, respectively, for up to 24 h at 37°C with 5% CO2.

P. zopfii cell internalization assay. Murine macrophages and bMECs were infected with P. zopfii for up to 8 h, washed with PBS (pH 7.4) and incubated for 2 h with gentamycin (200 μg/mL) to eliminate extracellular P. zopfii. Cells were washed with PBS to eliminate non-adherent bacteria and then lysed with 0.5% Triton X-100 (v/v) to determine CFU by 10-fold serial dilution. Further confirmation of phagocytic activity of macrophages was done by actin inhibition (cytochalasin D; C8273, Sigma, USA; 1 h) before inoculation.

Protein determination of apoptotic cytochrome-c, caspase-9, and caspase-3. Proteins from bMECs or homogenized murine mammary tissue were size-separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane (0.45 μm) (Millipore Sigma, Gillingham, Dorset, UK). Membrane was blocked with 5% skim milk in TBS-T (150 mM NaCl, 10 mM Tris base, 0.05% Tween 20, pH 7.4)
at room temperature for 120 min and then incubated overnight at 4°C with primary antibodies for caspase-9 (Cat # ab69514, Abcam USA), caspase-3 (Cat # ab90437, Abcam USA), cytochrome-c (Cat # ab110325, Abcam USA) and housekeeping β-tubulin (Cell Signaling Technology, Danvers, MA, USA). The membrane was rinsed with TBS-T and incubated with HRP-labeled secondary goat anti-rabbit IgG (ZRA03, Biotech, China) or goat anti-mouse IgG (ZM03, Biotech, China) at 37°C for 60 min. Signals were detected using enhanced chemiluminescence (Cat # PE0010, Solarbio Life Sciences, Beijing, China).

Protein detection by ELISA. Secreted Cxcl-1 and TNF-α proteins in infected and control mice were quantified by ELISAs (DuoSet ELISA # DY453–05 and # DY410–05, R&D Systems, Minneapolis, MN, USA).
Statistical analyses. Data were analyzed in triplicate for reproducibility and were expressed as mean ± standard deviation (SD). Data from infected and uninfected groups were analyzed using a paired Student’s t-test with a 95% confidence interval. Data were further analyzed by ANOVA and post hoc tests using SPSS 20.0 (International Business Machines Corporation, Armonk, NY, USA). For all analyses, *P < 0.05, **P < 0.01.

Results

P. zopfii GT-II induced mastitis and apoptosis in a mouse model. To investigate causative effects of P. zopfii GT-II in protothecal mastitis, lactating mice were intramammary challenged with P. zopfii GT-II isolated from a bovine clinical mastitis case. Round to oval sporangia with regular internal divisions compatible with P. zopfii were observed in the mammary gland of lactating mice at 4 dpi, as detected by PAS and GMS staining (Fig. 1A). Prototheca zopfii GT-II replicated in the murine mammary gland as it was recovered by culture in greater amounts at 4 dpi compared to the initial inoculum (mean 3.4 × 10⁷ CFU/g tissue).

Prototheca zopfii GT-II induced acute mastitis with infiltration of leukocytes throughout the parenchyma and within lumina of alveoli. Prototheca zopfii GT-II were present both free within alveolar lumina and throughout the interstitium of the mammary tissue (Fig. 1A). Using immune detection, macrophages were demonstrated in the mammary interstitium and neutrophils diffusely distributed in P. zopfii GT-II-infected mice (Fig. 1A). The presence of P. zopfii GT-II upregulated gene activity and protein production of pro-inflammatory TNF-α, IL-1β and Cxcl-1 in mammary tissue at 4 dpi (Fig. 1B,C).

Next, we determined whether intramammary infection with P. zopfii GT-II involved apoptosis and oxidative stress, as described in cultured bovine mammary epithelial cells (bMECs)²⁰,²¹. Apoptotic cells were quantified at 4 dpi with P. zopfii GT-II (Fig. 1D). Transcriptional analysis demonstrated that mRNA expression of caspase-9 and caspase-3 genes regulating mitochondrion-mediated apoptosis was higher in P. zopfii GT-II infected mice (Fig. 1E) with cleavage of caspase-3 protein (Fig. 1F). Expression of Bax gene increased in mammary tissue after P. zopfii GT-II inoculation (Supplementary Fig. 2A), whereas expression of Bcl-2 decreased (Fig. 1E). Expression of cytochrome-c released into the cytosol to trigger apoptosis (Fig. 1E) and Apaf-1 also increased in P. zopfii GT-II inoculated mice (Supplementary Fig. 2B).

P. zopfii GT-II-driven apoptosis occurred in both mammary epithelial cells and macrophages. Since mastitis is a process involving epithelial cells and leukocytes, we investigated contributions of single-cell components in pathogenesis of P. zopfii GT-II mastitis and apoptotic responses. We used a murine MEC (HC11) with ability to produce milk proteins (beta-casein) in response to prolactin²⁸. Infection with P. zopfii GT-II in MEC induced early IL-1β, TNF-α and Cxcl-1 gene expression (after 2 hpi) (Fig. 2A–C). Apoptotic cells appeared...
Later (24 hpi; Fig. 2D) with an increased transcriptional expression of hallmark apoptotic genes, Bax, Apaf-1 (Supplementary Fig. 2C,D), cytochrome-c, caspase-9 and caspase-3 genes (Fig. 2E–G). Expression of Bcl-2 was reduced (Fig. 2H).

To examine the role of macrophages, key in chronic mastitis29, murine macrophages (J774) with phagocytic characteristics were challenged with P. zopfii GT-II. Prototheca zopfii GT-II internalized inside macrophages in a time-dependent fashion (up to 8 hpi; Fig. 3A). This internalization seemed to be an active microbe process (P. zopfii dependent) rather than a phagocytic event, as actin inhibition in macrophages (by cytochalasin D) did not prevent P. zopfii GT-II internalization (Fig. 3A). Infection of P. zopfii GT-II in macrophages upregulated mRNA expression of IL-1β, TNF-α and Cxcl-1 (2 h; Fig. 3B–D). In contrast, TNF-α expression decreased over time (Fig. 3C). Prototheca zopfii GT-II induced apoptosis in macrophages as detected by TUNEL assay, with more cell death at 12 and 24 hpi (Fig. 3E) and upregulated expression of Bax, Apaf-1 (Supplementary Fig. 2E,F), cytochrome-c, caspase-9, and caspase-3 genes, whereas Bcl-2 expression decreased in a time-dependent manner (Fig. 3F–I).

P. zopfii GT-II induced apoptosis in bovine mammary epithelial cells. To verify apoptotic effects of P. zopfii in the target animal species (cattle), prototype bovine MECs with morphological and functional characteristics of normal mammary epithelial cells were challenged with P. zopfii GT-II and GT-I common commensals in farm environments (e.g., animal bedding, soil). Prototheca zopfii GT-I did not induce any apoptotic effects, but P. zopfii GT-II caused TUNEL-mediated apoptosis in a time-dependent manner (Fig. 4A). This occurred rapidly, as P. zopfii GT-II were internalized by bMECs in the first 4 hpi, as confirmed by culture (Fig. 4B) and TEM (Fig. 4C). Apoptotic effects induced by P. zopfii GT-II were likely of mitochondrial origin, as mitochondrial transmembrane depolarization was detected by immunofluorescence and flow cytometry (12–24 hpi; Fig. 4D–E).

Transcriptional expression of genes regulating mitochondrion-mediated apoptosis, including increased Bax and Apaf-1 (Supplementary Fig. 2G,H) and decreased Bcl-2, were detected in bovine MECs inoculated with P. zopfii GT-II (Fig. 5A). Expression of caspase-9 mRNA at early points (4 hpi) followed by caspase-3 mRNA later (24 hpi), increased after P. zopfii GT-II infection (Fig. 5B,C). Likewise, cytochrome-c and cleaved caspase-9 and-3 were over time increasingly immune blotted (Fig. 5D) and immunolocalized (Fig. 5E) in bMECs infected with P. zopfii GT-II. Apart from decreased Bcl-2 expression after 24 hpi, no effect of P. zopfii GT-I on apoptotic genes in bMECs was observed (Fig. 5A).

Figure 3. Murine macrophages infected with P. zopfii GT-II. (A) Internalization of P. zopfii GT-II in mouse macrophages in time-dependent manner, with and without cytochalasin D. (B–D) Level of cytokines (TNF-α, IL-1β and Cxcl-1) in murine macrophages. (E) TUNEL assay of mouse macrophages, quantitative analysis of apoptotic positive cells TUNEL positive apoptotic cells (20×). (F–I) Transcriptomic expression of Bcl-2, cytochrome-c, caspase-9, and caspase-3 at 4, 12 and 24 h after infection with P. zopfii GT-II in mouse macrophages on qPCR analysis and expressed as fold change relative to uninfected cells. Data are mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.
Infection of *P. zopfii* GT-II induced pro-inflammatory responses in bMECs as demonstrated by upregulated mRNA expression of *IL-1β*, *TNF-α* and *IL-8* (after 2 hpi; Fig. 5F, H). However, GT-I did not modify any inflammatory cytokine response in bMECs and demonstrated the apthogenic nature of this *Prototheca*. Taken together, *P. zopfii* GT-II was demonstrated to cause udder disease by provoking apoptosis and inducing inflammatory cytokine expression in mammary epithelium.

**Discussion**

Previously, pathogenesis of protothecal mastitis and virulence of *P. zopfii* GT-II isolated from bovine milk were uncertain. In this study, we used a *Prototheca spp.* identified as *P. zopfii* GT-II following a taxonomic approach commonly accepted for *Prototheca* and a cytb-based genotyping used for unambiguous *Prototheca spp.* identification based on the protothecal phylogeny and we described the pathogenic role of *P. zopfii* GT-II when initiating acute mastitis and mitochondrion-mediated apoptosis using a murine mastitis model and cultured mammary epithelial cells and macrophages. Our study demonstrated that *P. zopfii* GT-II invaded mammary parenchyma and caused acute mastitis, with severe infiltration of macrophages and neutrophils and marked epithelial damage. A destructive role of *P. zopfii* GT-II has been reported in the udder interstitium of cows and mammary acini of mice experimentally infected with *P. zopfii* GT-II.

Mammary epithelial cells are essential in microbial infection for sensing pathogens and producing an array of inflammatory cytokines. Pro-inflammatory cytokines, including *TNF-α*, *IL-1β*, *IL-6* and *IL-8*, have direct cytopathic effects leading to tissue damage. Additionally, *IL-1β* and *TNF-α* can induce cell apoptosis. *Prototheca zopfii* GT-II infection triggered expression of *IL-1β*, *Ccl-1(IL-8)*, and *TNF-α* in murine macrophages and bMECs. Thus, *P. zopfii* GT-II provoked apoptosis of bMECs by inducing *IL-1β* and *TNF-α* release in macrophages and mammary epithelial cells. *Prototheca zopfii* GT-II was more pathogenic than *P. zopfii* GT-I, commonly isolated as an environmental apathogenic microbe. *Prototheca zopfii* GT-II induced more *IL-8* mRNA in bMECs compared to GT-I-inoculated or uninfected cells. Increased levels of *IL-8* mRNA in murine MECs and bovine MECs induced by *P. zopfii* GT-II demonstrated that mammary epithelial cells are an important source of *IL-8* and that this chemokine is key during protothecal mastitis, perhaps by recruiting leukocytes, as demonstrated by its chemoattractant role in *Staphylococcus aureus* infection in bMECs.
Figure 5. In vitro infection of bovine mammary epithelial cells (bMECs) with *Prototheca zopfii* genotype (GT)-I and -II. (A–C) Transcriptomic analysis of Bcl-2, caspase-9 and caspase-3, respectively. (D,E) Western blot and confocal laser scanning microscopic analysis of cytochrome-c, caspase-9 and caspase-3 in bMECs, in western blot each samples run on two gels, for control and respective target antigen and cropped according to size of antibodies. (F,H) mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-8) quantified by qPCR in bMECs after infection of *Prototheca zopfii* genotype -I and -II infection. *P < 0.05, **P < 0.01.

Figure 6. Schematic presentation of mitochondrial-caspase induced apoptosis and inflammation. Depolarization of mitochondrial transmembrane ($\Delta\Psi_m$) causes the release of cytochrome-c, which may initiate caspase cascade. Cytochrome-c bonds with apoptotic protease-activating factor 1 (Apaf-1) and activates caspase-9, this cleaves and activates caspase-3, which triggers apoptosis. NF-κB subunit 65 transiting into the nucleus wherein it regulates transcription of pro-inflammatory genes, e.g. IL-1β and TNF-α.
Whereas P. zopfii has been reported to induce apoptosis in cultured bMECs\textsuperscript{21,22}, we demonstrated the pro-apoptotic role of P. zopfii GT-II in a murine mastitis model. The pro-apoptotic character of P. zopfii GT-II was demonstrated by increased numbers of TUNEL-positive cells in P. zopfii GT-II-infected mice, along with reduced Bel-2 mRNA and elevated transcriptomic levels of Bax, Apaf-1, caspase-3, and caspase-9. These all indicated apoptosis via the intrinsic pathway, with functional alterations in mitochondria in mammary epithelial cells infected with P. zopfii GT-II. Moreover, P. zopfii GT-II induced ROS generation\textsuperscript{21} which triggers mitochondrial Bax, a proapoptotic element of the Bel-2 family proteins\textsuperscript{37}. Prototheca zopfii GT-II invaded bMECs and murine macrophages, and indeed, apoptotic effects were promoted by microbial internalization, but independent of phagocytosis. Prototheca zopfii GT-II had higher penetration capabilities in bMECs than P. zopfii GT-I. We propose that mitochondrial damage due to P. zopfii GT-II invasion released protein cytochrome-c from intermembrane spaces into cytosol, which bonded with Apaf-1 to initiate apoptosis formation and activation of caspase-9 and caspase-3\textsuperscript{40-49}. Such P. zopfii-driven apoptosis was not restricted to mammary epithelial cells but also applied to leukocytes, including murine macrophages. Whereas P. zopfii GT-II was a pathogenic type of Prototheca causing mastitis, studies with other Prototheca strains may elucidate the complexity of these algae and their interactions with host and environment. A hypothetical schematic illustration of mitochondrial caspase-induced apoptotic pathway and NF-κB subunit 65 transiting into the nucleus in protothecal mastitis (Fig. 6) was consistent with reports in bMECs, wherein P. zopfii GT-II regulated transcription of pro-inflammatory genes like IL-1β and TNF-α\textsuperscript{35}. In conclusion, pathomorphological alteration caused by P. zopfii GT-II highlighted this genotype as a mastitis pathogen capable of penetrating into mammary epithelial cells to induce inflammation and cell death, via mitochondrial-dependent apoptosis.

Received: 20 March 2019; Accepted: 6 January 2020;
Published online: 20 January 2020

References

1. Mushqat, S. et al. Bovine mastitis: An appraisal of its alternative herbal cure. Microb. Pathog. 114, 357–361 (2018).
2. Milanov, D., Petrović, T., Polače, V., Suvadjić, L. & Bojkovski, J. Mastitis associated with Prototheca zopfii - An emerging health and economic problem on dairy farms. J. Vet. Res. 60, 373–378 (2016).
3. Irgang, A., Murugaiyan, J., Weise, C., Azab, W. & Roesler, U. Well-known surface and extracellular antigens of pathogenic microorganisms among the immunodominant proteins of the infectious microalgae Prototheca zopfii. Front. Cell. Infect. Microbiol. 5, 67 (2015).
4. Krukowski, H., Lisowski, A. & Nowakowicz-Dąbrowska, K. Prototheca zopfii, Bovine mastitis pathogen capable of penetrating into mammary epithelial cells to induce inflammation and cell death, via mitochondrial-dependent apoptosis. Sci. Rep. 8, 120 (2018).
5. Jagielski, T. et al. Cyto as a new genetic marker for differentiation of Prototheca species. J. Clin. Microbiol. https://doi.org/10.1128/JCM.00584-18 (2018).
6. Sabokhawa, H. et al. Short communication: Molecular typing of Prototheca zopfii from bovine mastitis in Japan. J. Dairy Sci. 95, 4442–4446 (2012).
7. Wawron, W., Bochniarz, M., Piech, T., Lopuszyński, W. & Wawron, W. Outbreak of protothecal mastitis in a herd of dairy cows in Poland. Bull. Vet. Inst. Pulawy 57, 335–339 (2013).
8. Bozzo, G. et al. Occurrence of Prototheca spp. in cow milk samples. New Microbiol. 37, 459–464 (2014).
9. Park, H. S., Chang Moon, D., Hyun, B. H. & Lim, S. K. Short communication: Occurrence and persistence of Prototheca zopfii in dairy herds of Korea. J. Dairy Sci. 102, 2539–2543 (2019).
10. Pieper, I. et al. Herd characteristics and cow-level factors associated with Prototheca mastitis on dairy farms in Ontario, Canada. J. Dairy Sci. 95, 5635–5644 (2012).
11. Suvadjić, B. et al. Molecular identification of Prototheca zopfii genotype 2 mastitis isolates and their influence on the milk somatic cell count. Vet. Arh. 87, 249–258 (2017).
12. Pal, M., Abraha, A., Rahman, M. T. & Dave, P. Protothecosis: An emerging algal disease of humans and animals. J. Vet. Res. 73, 117–119 (2011).
13. Da Costa, E. O. et al. Genotypes isolated from cases of bovine mastitis and cow barns in China. J. Clin. Microbiol. 57, 106–108 (2013).
14. Abdelhameed, K. G. Detection of Prototheca zopfii genotypes isolated from bovine mastitis in Japan. Vet. Microbiol. 183, 181–187 (2016).
15. Shahid, M. et al. Characterization of Prototheca zopfii genotypes isolated from cases of bovine mastitis and cow barns in China. Mycopathologia 181, 185–195 (2016).
16. Osumi, T. et al. Prototheca zopfii genotypes isolated from cow barns and bovine mastitis in Japan. Vet. Microbiol. 131, 419–423 (2008).
17. Shahid, M. et al. Characterization of Prototheca zopfii genotypes isolated from cases of bovine mastitis and cow barns in China. Mycopathologia 181, 185–195 (2016).
18. Jagielski, T. et al. A survey on the incidence of Prototheca mastitis in dairy herds in Lublin province, Poland. J. Dairy Sci. 102, 619–628 (2019a).
19. Jagielski, T. et al. The genus Prototheca (Trebouxiophyceae, Chlorophyta) revisited: Implications from molecular taxonomic studies. Algal Res. 43, 101639 (2019b).
20. Shahid, M. et al. Prototheca zopfii isolated from bovine mastitis induced oxidative stress and apoptosis in bovine mammary epithelial cells. Oncotarget 8, 31938–31947 (2017).
21. Shahid, M. et al. Prototheca zopfii induced ultrastructural features associated with apoptosis in bovine mammary epithelial cells. Front. Cell. Infect. Microbiol. 7, 299 (2017).
22. Gao, J. et al. Characterization of Prototheca zopfii associated with outbreak of bovine clinical mastitis in herd of Beijing, China. Mycopathologia 173, 275–281 (2012).
23. Möller, A., Truyen, U. & Roesler, U. Prototheca zopfii genotype 2—The causative agent of bovine protothecal mastitis? Vet. Microbiol. 120, 370–374 (2007).
24. Seok, J. Y. et al. Human cutaneous protothecosis: report of a case and literature review. Korean J. Pathol. 47, 575–578 (2013).
25. Pereyra, E. A. L. et al. Detection of Staphylococcus aureus adherence and biofilm-producing genes and their expression during internalization in bovine mammary epithelial cells. Vet. Microbiol. 183, 69–77 (2016).
40. Xu, Z. et al. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

39. Tait, S. W. G. & Green, D. R. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat. Rev. Mol. Cell Biol. 11, 621–632 (2010).

38. Chen, W. et al. Interleukin-1β and tumor necrosis factor (TNF)-α sensitize human thyroid epithelial cells to TNF-related apoptosis-inducing ligand-induced apoptosis through increases in procaspase-7 and bid, and the down-regulation of p44/42 mitogen-activated protein kinase activity. J. Clin. Endocrinol. Metab. 89, 250–257 (2004).

37. Li, Z., Meng, J., Xu, T. J., Qin, X. Y. & Zhou, X. D. Sodium selenite induces apoptosis in colon cancer cells via Bax-dependent mitochondrial pathway. Eur. Rev. Med. Pharmacol. Sci. 17, 2166–2171 (2013).

36. Kiku, Y. et al. The cell wall component lipoteichoic acid of Staphylococcus aureus induces chemokine expression in bovine mammary epithelial cells. J. Vet. Med. Sci. 78, 1505–1510 (2016).

35. Deng, Z. et al. An investigation of the innate immune response in bovine mammary epithelial cells challenged by Prototheca zopfii. Mycopathologia 181, 823–832 (2016).

34. Mezosi, E. et al. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

33. Hristova, M., Yordanov, M. & Ivanovska, N. Effect of fangchinoline in murine models of multiple organ dysfunction syndrome and septic shock. Inflamm. Res. 52, 1–7 (2003).

32. Liu, M. et al. Interleukin-1β and tumor necrosis factor (TNF)-α sensitize human thyroid epithelial cells to TNF-related apoptosis-inducing ligand-induced apoptosis through increases in procaspase-7 and bid, and the down-regulation of p44/42 mitogen-activated protein kinase activity. J. Clin. Endocrinol. Metab. 89, 250–257 (2004).

31. Chang, R. et al. Treatment with gentamicin on a murine model of protothecal mastitis. Mycopathologia 175, 241–248 (2013).

30. Bueno, V. F. F. et al. Treatment with gentamicin on a murine model of protothecal mastitis. Mycopathologia 175, 241–248 (2013).

29. Guerrero, I. et al. Host responses associated with chronic staphylococcal mastitis in rabbits. Vet. J. 204, 338–344 (2015).

28. Ball, R. K., Friis, R. R., Schoenenberger, C. A., Doppler, W. & Groner, B. Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. EMBO J. 7, 2089–2095 (1988).

27. Bustin, S. A. et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622 (2009).

26. Poult. Sci. 95, 2405–2413 (2016).

25. Chang, R. et al. The effect of the mitochondrial permeability transition pore on apoptosis in Eimeria tenella host cells. Poult. Sci. 95, 2405–2413 (2016).

24. Deng, Z. et al. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

23. Hristova, M., Yordanov, M. & Ivanovska, N. Effect of fangchinoline in murine models of multiple organ dysfunction syndrome and septic shock. Inflamm. Res. 52, 1–7 (2003).

22. Kiku, Y. et al. The cell wall component lipoteichoic acid of Staphylococcus aureus induces chemokine expression in bovine mammary epithelial cells. J. Vet. Med. Sci. 78, 1505–1510 (2016).

21. Li, Z., Meng, J., Xu, T. J., Qin, X. Y. & Zhou, X. D. Sodium selenite induces apoptosis in colon cancer cells via bax-dependent mitochondrial pathway. Eur. Rev. Med. Pharmacol. Sci. 17, 2166–2171 (2013).

20. Chem, W. et al. Nocardia cyriacigeogica from bovine mastitis induced in vitro apoptosis of bovine mammary epithelial cells via activation of mitochondrial-caspase pathway. Front. Cell. Infect. Microbiol. 7, 194 (2017).

19. Tait, S. W. G. & Green, D. R. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat. Rev. Mol. Cell Biol. 11, 621–632 (2010).

18. Xu, Z. et al. The effect of the mitochondrial permeability transition pore on apoptosis in Eimeria tenella host cells. Poult. Sci. 95, 2405–2413 (2016).

17. Interleukin-1β and tumor necrosis factor (TNF) sensitize human thyroid epithelial cells to TNF-related apoptosis-inducing ligand-induced apoptosis through increases in procaspase-7 and bid, and the down-regulation of p44/42 mitogen-activated protein kinase activity. J. Clin. Endocrinol. Metab. 89, 250–257 (2004).

16. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

15. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

14. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

13. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

12. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

11. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

10. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

9. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

8. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

7. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

6. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

5. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

4. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

3. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

2. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).

1. The protective effect of caffeic acid against inflammation injury of primary bovine mammary epithelial cells induced by lipopolysaccharide. J. Dairy Sci. 97, 2856–2865 (2014).