Next-generation Sequencing Analysis of Bacterial Flora in Bovine Prototheca Mastitic Milk

Rui Kano¹, Yuki Kobayashi¹, Akitoyo Nishikawa¹, Ryo Murata¹, Takuya Itou², Takaaki Ito³, Kazuyuki Suzuki⁶ and Hiroshi Kamata¹

¹Department of Veterinary Pathobiology, Nihon University College of Bioresource Sciences
²Department of Preventive Veterinary Medicine and Animal Health, Nihon University College of Bioresource Sciences
³Onbetsu Shiranuka Livestock Veterinary Clinic, Kushiro Western Part Business Center
⁴Veterinary Bacteriology, Department of Pathobiology, School of Veterinary Medicine, Rakuno Gakuen University
⁵Veterinary Hospital Aichi P.F.A.M.A.
⁶Department of Large Animal Clinical Sciences, School of Veterinary Medicine, Rakuno Gakuen University

Abstract

Prototheca zopfii is an achlorophyllic algae that causes bovine mastitis, resulting in a reduction in milk production and the secretion of thin, watery milk with white flakes. The aim of the present study was to evaluate the bacterial flora in the udder environment in protothecal mastitis. We used metagenomic next-generation sequencing (NGS) analysis to identify 16S rRNA genes from bacterial flora present in milk samples from protothecal mastitic dairy cows.

Seven clinical strains of P. zopfii genotype 2 were isolated from 7 milk samples from 7 cases (Holstein cow) of protothecal mastitis; another 9 milk samples were obtained from 9 normal Holstein cows. The samples were collected in 2017 from cows in one dairy located in the Kushiro region in Hokkaido, Japan, which had a history of protothecal mastitis infection. The NGS produced 10,000 to 15,000 sequences in each DNA sample. To facilitate comparison, we grouped the sequencing results according to the culture-based protothecal mastitis diagnosis; sequences derived from the milk samples obtained from healthy cows were grouped separately.

Sequences classified as Streptococcus spp., Pseudomonas spp. and Sphingomonas spp., Caulobacter segnis, Macrococcus caseolyticus, Methylobacterium tarhaniae, and Sphingomonas leidyi were the main sequences detected in the groups of samples from cows characterized by culture as having protothecal mastitis. Notably, Calothrix desertica (a cyanobacterium) sequences showed higher prevalence in these samples.

To our knowledge, this is the first study to report that C. desertica sequences, effectively absent in the samples derived from healthy cows, are detected at high prevalence in samples from protothecal mastitic animals.

Key words: bacterial flora, bovine Prototheca mastitis, next-generation sequencing, Prototheca zopfii

Introduction

The genus Prototheca consists of achlorophyllic algae that are ubiquitous in cow-barn environments. Prototheca zopfii and P. blaschkeae are associated with bovine mastitis, which causes a reduction in milk production and secretion of thin, watery milk with white flakes⁷⁻⁷. Most cases of bovine protothecal mastitis are chronic and subclinical infections; and no effective treatment is known. Affected cows must be clearly identified and placed last in the milking order until the infected animals are culled. Bovine protothecal mastitis is therefore an important disease responsible for economic losses in the dairy industry. However, neither the infection mechanism nor the route of infection leading to mastitis have been defined, precluding prevention of this disease.

Recent studies have used metagenomics of bacterial 16S rRNA genes to investigate bacterial DNA diversity in milk samples from mastitic and healthy dairy cows, using the results to compare the bacterial flora in the udder environment.
between infected and uninfected animals. Notably, Oikonomou et al. reported that anaerobic bacteria that are known pathogens were detected in most of the bacterial mastitic milk samples regardless of the results of metagenomic analyses of 16S rRNA obtained by pyrosequencing\(^5\). The authors suggested that metagenomic analysis is an important tool for understanding the pathogenesis of bovine mastitis and could be further developed as a diagnostic tool.

The aim of the present study was to evaluate bacterial flora in the udder environment. To this end, we used metagenomic next-generation sequencing (NGS) to identify 16S rRNA genes of bacterial flora in milk samples from protothecal mastitic dairy cows. To the best of our knowledge, NGS analysis of the 16S rRNA gene has not been used previously to investigate milk samples from protothecal mastitic Holstein dairy cows.

**Materials and methods**

**Milk samples**

A total of 16 milk samples were subjected to metagenomic NGS of bacterial 16S rRNA genes. Milk samples, which were collected in 2017, were obtained by combining milk from the 4 teats of each animal; all were Holstein cows that were housed at a single dairy with a history of Protothecal mastitis and located in the Kushiro region in Hokkaido, Japan. Seven clinical strains of *P. zopfii* genotype 2 were isolated from 7 milk samples from 7 separate cases of protothecal mastitis; the other 9 milk samples were obtained from 9 normal (non-mastitic) animals.

All cases of bovine protothecal mastitis in this study were chronic and subclinical, and yielded positive results in culture tests and SCCs (Somatic Cell Counts) of milk samples\(^5,7\). Isolation and molecular typing procedures were described in our previous studies\(^5,7\).

**PCR amplification bacterial 16S rRNA genes**

Isolation of bacterial genomic DNA from milk was performed using a MORA-EXTRACT kit (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) according to the manufacturer’s instructions.

The V3-4 region of bacterial 16S rRNA genes were PCR-amplified from each sample using a composite pair of primers containing unique 17- or 21-base adapters, which were used to tag the PCR products from the respective samples (Illumina, Inc., San Diego, CA)\(^8\). The forward primer sequence was 5’-TCGTCCGCAAGGCTACATCTATGATATATAAGACAGCCTAGGGNGGGCTAGAG-3’, where the bold sequence is the forward primer’s overhanging adapter, and the italicized sequence is the universal broadly conserved bacterial primer S-D-Bact-0341-b-S-17 (in which N corresponds to any nucleotide, and W corresponds to A or T). The reverse primer sequence was 5’-GTCTCGTGGGCTCAGATGTGTA-3’. where the bold sequence is the reverse primer’s overhanging adapter, and the italicized sequence is the broad-range bacterial primer S-D-Bact-0907-a-A-20 (in which H corresponds to A, C, or T, and V corresponds to A, G, or C).

Primers S-D-Bact-0341-b-S-17 and S-D-Bact-0907-a-A-20 amplified approximately 460-bp fragments of the bacterial 16S rRNA genes (Illumina).

Genomic DNA samples (100 ng/sample) were subjected to amplification by PCR in a total volume of 30 ml, using a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\(_2\), 0.001% gelatin, 200 mM of each deoxynucleoside triphosphate, 1.0 unit of Taq polymerase (Takara Bio, Kyoto, Japan), and 50 mmol each of a pair of primers. Amplification was carried out over 35 cycles, each consisting of template denaturation (1 min, 94°C), primer annealing (1 min, 63°C), and polymerization (2 min, 72°C). PCR products were detected by electrophoresis of an aliquot on a 2% agarose gel followed by staining with ethidium bromide and visualization under UV light.

**16S metagenomic sequencing**

PCR amplicons were purified with a Nucleospin® Gel and PCR Clean-up (Takara Bio). DNA concentration and purity were evaluated by optical density using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific K.K., Kanagawa, Japan), and the final concentrations of the samples were adjusted to 4 nmol per 5 ml.

The 16S metagenomic sequence library preparation was performed using the NEBNext Ultra II DNA library prep kit (New England BioLabs Inc, Ipswich, MA).

The library of the V3-4 region of bacterial 16S rRNA genes was sequenced using the MiSeq\(^\text{TM}\) System (Illumina).

To facilitate a detailed (species-level) analysis of the sequences, we performed the following steps. 10,000 to 15,000 sequences from each sample were used to create a new FASTAQ sequence file. This file was trimmed to remove the 16S primers and filtered to remove additional sequences of low-quality. The resulting file was uploaded to the OTU (Operational Taxonomic Unit) picking tool (World Fusion US, Inc., San Diego, CA; http://www.w-fusionus.com/metagenome-kin), and then processed using the complete linkage clustering tool (which clustered the aligned sequences into OTUs). Finally, the dereplicate function was used to create one representative sequence for each OTU. A new file of representative sequences was then created. Homology analysis with the Basic Local Alignment Search Tool (BLAST algorithm) for bacterial 16S was performed using the Metagenome@Kin (World Fusion US, Inc.).

NGS produced 10,000 to 15,000 sequences from each DNA sample. To facilitate comparison, we grouped the sequencing results according to the culture-based protothecal mastitis diagnosis; sequences derived from the milk samples obtained
from healthy cows were grouped separately. Nucleotide sequence identity for bacterial species ranged from 98% to 100% homology among sequence samples examined in the present work, including the reference sequences of bacterial 16S in the BLAST database.

**Results**

The most frequent bacterial sequences detected by NGS in milk samples obtained from protothecal mastitic and healthy cows are shown in Table 1.

Sequences classified by Metagenome@Kin as *Streptococcus* spp., *Pseudomonas* spp. and *Sphingomonas* spp., *Caulobacter segnis*, *Macrococcus caseolyticus*, *Methylobacterium tarhaniae*, and *Sphingomonas leidyi* were the most prevalent sequences in the groups of samples characterized by culture as protothecal mastitis (Table 1). Notably, *Calothrix desertica* (a cyanobacterium) showed higher prevalence in these samples than those from healthy cows (Table 1).

**Discussion**

To our knowledge, the present study is the first demonstration that *C. desertica* sequences, which were effectively absent in samples derived from healthy animals, were highly prevalent in samples from protothecal mastitic cows. *C. desertica* is a cyanobacterium (formerly considered a blue-green algae) that is widely distributed in the natural environment (AlgaeBase: http://www.algaebase.org/search/species/detail/?species_id=134352). A role for this species in the pathogenesis of bovine mastitis has not been reported previously. Oikonomou et al. reported that anaerobic bacteria

| Protothecal mastitic milk | Cow No. 30 Bacterial species | %* | Cow No. 31 Bacterial species | %* |
|---------------------------|------------------------------|----|-----------------------------|----|
| *Intestinimonas butyriciproducens* | 4.27 | *Streptococcus parauberis* | 30.4 |
| *Lactobacillus crispatus* | 3.82 | *Streptococcus dysgalactiae* | 13.4 |
| *Romboutsia ilealis* | 3.3 | *Sphingomonas leidyi* | 11 |
| *Bacteroides plebeius* | 2.92 | *Calothrix desertica* | 9.74 |
| *Sphingomonas leidyi* | 2.81 | *Methylobacterium tarhaniae* | 3.66 |
| *Clostridium straminisolvens* | 2.81 | *Caulobacter segnis* | 3.22 |
| *Caulobacter segnis* | 2.66 | *Pseudomonas corrugata* | 1.39 |
| *Sporobacter termitidis* | 2.51 | *Clostridium straminisolvens* | 1.07 |
| *Papillibacter cinnamivorans* | 2.25 | *Sphingomonas paucimobilis* | 1.04 |
| *Aerococcus viridans* | 1.95 | *Intestinimonas butyriciproducens* | 0.99 |
| **Total** | 29.3 | **Total** | 75.91 |
| **Unclassified** | 70.7 | **Unclassified** | 24.09 |

| Protothecal mastitic milk | Cow No. 32 Bacterial species | %* | Cow No. 36 Bacterial species | %* |
|---------------------------|------------------------------|----|-----------------------------|----|
| *Psychrobacter pulmonis* | 22.6 | *Streptococcus parauberis* | 40.8 |
| *Calothrix desertica* | 13.2 | *Macrococcus caseolyticus* | 8.62 |
| *Psychrobacter maritimus* | 9.48 | *Psychrobacter pulmonis* | 7.21 |
| *Aerococcus viridans* | 6.56 | *Acinetobacter indicus* | 4.42 |
| *Macrococcus caseolyticus* | 4.38 | *Empedobacter falsenii* | 4.27 |
| *Streptococcus parauberis* | 3.87 | *Psychrobacter maritimus* | 3.97 |
| *Psychrobacter submarinus* | 3.16 | *Acinetobacter lwoffii* | 3.49 |
| *Psychrobacter adeliensis* | 2.52 | *Aerococcus viridans* | 2.15 |
| *Phascolarctobacterium succinatutens* | 2.31 | *Escherichia fergusonii* | 2.08 |
| *Intestinimonas butyriciproducens* | 1.29 | *Psychrobacter submarinus* | 1.78 |
| **Total** | 69.37 | **Total** | 78.79 |
| **Unclassified** | 30.63 | **Unclassified** | 21.21 |
### Cow No. 37
| Bacterial species                  | %*  |
|------------------------------------|-----|
| Calothrix desertica                | 63.6|
| Sphingomonas leidyi                | 11.84|
| Caulobacter segnis                 | 4.37|
| Methylobacterium tarhaniae         | 4.1 |
| Intestinimonas butyriciproducens   | 1.55|
| Asaccharospora irregulareis        | 0.82|
| Bacteroides plebeius               | 0.74|
| Methylobacterium radiotolerans     | 0.68|
| Sporobacter termitidis             | 0.66|
| Paludibacter propionicigenes       | 0.62|
| **Total**                          | 88.98|
| Unclassified⁺                     | 11.02|

### Cow No. 44
| Bacterial species                  | %*  |
|------------------------------------|-----|
| Calothrix desertica                | 32.86|
| Streptococcus parauberis           | 26.73|
| Intestinimonas butyriciproducens   | 2.17|
| Paraprevotella clara               | 1.28|
| Clostridium straminisolvens        | 1.14|
| Clostridium cellubiovarum          | 0.99|
| Macroccus caseolyticus             | 0.94|
| Sporobacter termitidis             | 0.94|
| Bacteroides plebeius               | 0.89|
| **Total**                          | 69.52|
| Unclassified⁺                     | 30.48|

### Cow No. 46
| Bacterial species                  | %*  |
|------------------------------------|-----|
| Calothrix desertica                | 7.41 |
| Pseudomonas fragi                  | 7.06 |
| Pseudomonas deceptionensis         | 5.12 |
| Atopostipes suclaoaclus            | 4.77 |
| Macroccus caseolyticus             | 3.5 |
| Intestinimonas butyriciproducens   | 3.42 |
| Phascolarctobacterium succinatutens| 2.88 |
| Sphingomonas leidyi                | 2.62 |
| Streptococcus parauberis           | 2.53 |
| Bacillus toyonensis                | 2.35 |
| **Total**                          | 41.66|
| Unclassified⁺                     | 58.34|

### Healthy cow milk

#### Cow No. 1
| Bacterial species                  | %*  |
|------------------------------------|-----|
| Sneathia sanguinegens              | 64.11|
| Streptobacillus moniliformis       | 15.03|
| Sphingomonas leidyi                | 2.5 |
| Intestinimonas butyriciproducens   | 1.34|
| Methylobacterium tarhaniae         | 1.17|
| Clostridium straminisolvens        | 1   |
| Treponema brennaborens             | 1   |
| Caulobacter segnis                 | 0.83|
| Acinetobacter lwofii               | 0.83|
| Prosthocobacter fluviiatis         | 0.83|
| **Total**                          | 88.64|
| Unclassified⁺                     | 11.36|

#### Cow No. 13
| Bacterial species                  | %*  |
|------------------------------------|-----|
| Sphingomonas leidyi                | 13  |
| Intestinimonas butyriciproducens   | 9.74|
| Phascolarctobacterium succinatutens| 5.68|
| Bacteroides plebeius               | 5.57|
| Clostridium straminisolvens        | 3.53|
| Caulobacter segnis                 | 3.53|
| Oscillibacter valericigenes        | 3.38|
| Sporobacter termitidis             | 2.86|
| Papillibacter cinnamivorans        | 2.67|
| Bacteroides xylanolyticus          | 2.34|
| **Total**                          | 52.3 |
| Unclassified⁺                     | 47.7 |

#### Cow No. 21
| Bacterial species                  | %*  |
|------------------------------------|-----|
| Sphingomonas leidyi                | 14.8 |
| Methylobacterium tarhaniae         | 5.14 |
| Sporobacter termitidis             | 4.53 |
| Turicibacter sanguinis             | 4.04 |
| Caulobacter segnis                 | 3.79 |
| Clostridium clostridioforme        | 3.55 |
| **Total**                          |      |

#### Cow No. 23
| Bacterial species                  | %*  |
|------------------------------------|-----|
| Psychrobacter palmonis             | 25.8 |
| Aerococcus viridans                | 18.7 |
| Psychrobacter maritimus            | 9.63 |
| Acinetobacter indicus              | 8.4 |
| Acinetobacter lwofii               | 7.2 |
| Psychrobacter submarinus           | 3.45 |
| Bacterial species                        | %*  | Bacterial species                        | %*  |
|------------------------------------------|-----|------------------------------------------|-----|
| Romboutsia ilealis                       | 3.49| Romboutsia ilealis                       | 2.74|
| Atopostipes suicoalcalis                 | 3   | Eubacterium tenue                        | 1.91|
| Eubacterium coprostanoligenes            | 2.94| Psychrobacter arcticus                   | 1.61|
| Acetivibrio ethanoligignens              | 2.08| Clostridium disporicum                   | 1.24|
| **Total**                                | 47.36| **Total**                                | 80.68|
| **Unclassified**                         | 52.64| **Unclassified**                         | 19.32|

### Cow No. 27

| Bacterial species                        | %*  |
|------------------------------------------|-----|
| Aerococcus viridans                      | 36.39|
| Sphingomonas leidyi                      | 11.01|
| Psychrobacter maritimus                  | 10.7 |
| Bifidobacterium pseudolongum             | 7.03 |
| Ruminococcus bromii                      | 4.59 |
| Caulobacter segnis                       | 4.59 |
| Syntrophococcus sacromutans              | 4.59 |
| Methanobrevibacter thaieri               | 2.75 |
| Butyribrio hungatei                      | 2.45 |
| Romboutsia ilealis                       | 2.45 |
| **Total**                                | 86.55|
| **Unclassified**                         | 13.45|

### Cow No. 28

| Bacterial species                        | %*  |
|------------------------------------------|-----|
| Acinetobacter lwoffii                    | 26.65|
| Aerococcus viridans                      | 25.06|
| Psychrobacter pulmonis                   | 9.38 |
| Corynebacterium callunae                 | 6.36 |
| Macrococcus caseolyticus                 | 5.7  |
| Acinetobacter indicus                    | 3.6  |
| Actinobacter radioreisitens              | 2.33 |
| Psychrobacter arcticus                   | 2.2  |
| Acinetobacter bouvetii                   | 2.01 |
| Aerococcus viridans                      | 1.25 |
| **Total**                                | 84.54|
| **Unclassified**                         | 15.46|

### Cow No. 29

| Bacterial species                        | %*  |
|------------------------------------------|-----|
| Sporobacter termitidis                   | 3.92 |
| Intestinimonas butyriciproducens         | 3.87 |
| Staphylococcus pseudintermedius          | 3.36 |
| Clostridium straminisolvens              | 2.96 |
| Sphingomonas leidyi                      | 2.9  |
| Papillibacter cinnamivorans              | 2.39 |
| Staphylococcus chromogenes               | 2.33 |
| Bacteroides plebeicus                    | 2.28 |
| Clostridium cellibrioparum               | 2.16 |
| Ethanoligenens harbinense                | 2.16 |
| **Total**                                | 28.33|
| **Unclassified**                         | 71.67|

### Cow No. 39

| Bacterial species                        | %*  |
|------------------------------------------|-----|
| Staphylococcus epidermidis               | 20.5 |
| Sphingomonas leidyi                      | 11.7 |
| Acinetobacter lwoffii                    | 6.22 |
| Acinetobacter calcoaceticus              | 4.31 |
| Caulobacter segnis                       | 3.96 |
| Actinobacter indicus                     | 3.3  |
| Methylobacterium tarhaeitae              | 3.22 |
| Acinetobacter bereziniae                 | 2.8  |
| Macrooccus caseolyticus                  | 2.56 |
| Psychrobacter pulmonis                   | 1.98 |
| **Total**                                | 60.55|
| **Unclassified**                         | 39.45|

### Cow No. 43

| Bacterial species                        | %*  |
|------------------------------------------|-----|
| Sphingomonas leidyi                      | 27.83|
| Methylbacterium tarhaeitae               | 9.57 |
| Caulobacter segnis                       | 7.92 |
| Clostridium straminisolvens              | 3.01 |
| Romboutsia ilealis                       | 2.76 |
| Sporobacter termitidis                   | 2.66 |
| Intestinimonas butyriciproducens         | 2.46 |
| Methylbacterium radiostoleras            | 2.21 |
| Phascolarctobacterium succinatatens      | 1.79 |
| Clostridium cellibrioparum               | 1.71 |
| **Total**                                | 61.92|
| **Unclassified**                         | 38.08|

* Percentage of the total number of identified 16S sequences in individual samples
+ Unclassified bacterial sequences
that are known pathogens are detected in most bacterial mastitic milk samples, regardless of the bacterial distribution defined by metagenomic analyses of sequences obtained through pyrosequencing of the 16S rRNA. However, the study did not report *C. desertica* in samples of bacterial mastitic or normal milk. Nonetheless, the study of Oikonomou et al., like the present study, indicated that changes in bacterial flora are typical in protothecal mastitis. *Prototheca* comprises achlorophyllic algae that are ubiquitous in cow-barn environments; infection by *Prototheca* species is considered an opportunistic infection that reflects alterations in the udder environment and host immunity compared to those of healthy cows.

In conclusion, the use of NGS of the 16S rRNA should be considered an important tool to advance our knowledge regarding the infection mechanism of bovine protothecal mastitis. With further development, this technique may find application as a preventive tool.

**Acknowledgments**

This study was supported by a Grant-in-Aid for Scientific Research ‘C’ (17K08110) and a grant (“International joint research and training of young researchers for zoonosis control in the globalized world”) from the Academic Frontier Project of the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT).

**Conflicts of interest**

The authors report no conflicts of interest. The authors alone were responsible for the content and writing of the paper.

**References**

1) Roesler U, Scholz H, Hensel A: Emended phenotypic characterization of *Prototheca zopfii*: a proposal for three biotypes and standards for their identification. Int J Syst Evol Microbiol 53: 1195-1199, 2003.
2) Roesler U, Möller A, Hensel A, Baumann D, Truyen U: Diversity within the current algal species *Prototheca zopfii*: a proposal for two *Prototheca zopfii* genotypes and description of a novel species, *Prototheca blaschkeae* sp. nov. Int J Syst Evol Microbiol 56: 1419-1425, 2006.
3) Möller A, Truyen U, Roesler U: *Prototheca zopfii* genotype 2: the causative agent of bovine protothecal mastitis? Vet Microbiol 120: 370-374, 2007.
4) Marques S, Silva E, Kraft C, Carvalheiro J, Videira A, Huss VA, Thompson G: Bovine mastitis associated with *Prototheca blaschkeae*. J Clin Microbiol 46: 1941-1945, 2008.
5) Osumi T, Kishimoto Y, Kano R, Maruyama M, Onozaki M, Makimura K, Ito T, Matsubara K, Hasegawa A: *Prototheca zopfii* genotypes isolated from cow barns and bovine mastitis in Japan. Vet Microbiol 131: 419-423, 2008.
6) Ricchi M, Goretti M, Branda E, Canmi G, Garbarino CA, Turchetti B, Moroni P, Arrigoni N, Buzzini P: Molecular characterization of *Prototheca* strains isolated from Italian dairy herds. J Dairy Sci 93: 4625-4631, 2010.
7) Sobukawa H, Yamaguchi S, Kano R, Ito T, Suzuki K, Onozaki M, Hasegawa A, Kamata H: Short communication: Molecular typing of *Prototheca zopfii* from bovine mastitis in Japan. J Dairy Sci 95: 4442-4446, 2012.
8) Oikonomou G, Machado VS, Santisteban C, Schukken YH, Bicalho RC: Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. PLoS One 7: e47671, 2012.
9) Bozzo G, Bonerba E, Di Pinto A, Bolzoni G, Ceci E, Mottola A, Tantillo G, Terio V: Occurrence of *Prototheca* spp. in cow milk samples. New Microbiol 37: 459-464, 2014.
10) Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FQ: Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41: e1, 2013.