Milk microRNA-146a as a potential biomarker in bovine tuberculosis

Milk microRNA-146a as a potential biomarker in bovine tuberculosis

Marco Iannaccone[1*], Gianfranco Cosenza[1*], Alfredo Pauciullo[2], Francesca Garofalo[3], Yolande T. Proroga[3], Federico Capuano[3], and Rosanna Capparelli[1].

[1] Department of Agriculture, University of Naples Federico II, via Università 100, 80055 Portici, Napoli, Italy.

[2] Department of Agricultural, Forest and Food Science, University of Torino, Grugliasco (TO), Italy

[3] Department of Food Microbiology, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Naples, Italy.

[*]Address for correspondence:

Marco Iannaccone,

Department of Agriculture, University of Naples Federico II, via Università 100,80055 Portici, Napoli, Italy.

Tel: +390812539276

Fax: +390812531730

E-mail: m.iannaccone@unina.it

Gianfranco Cosenza,

Department of Agriculture, University of Naples Federico II, via Universita 100,80055 Portici, Napoli, Italy.

Tel: +3908125339272

Fax: +390812531730

E-mail: giacosen@unina.it

Summary

In this research communication we exploited the potential use of milk microRNAs (miRs) as biomarker for bovine tuberculosis (bTB). bTB is a zoonotic disease caused by *Mycobacterium bovis* which affects animal health influencing herd economic sustainability. Diagnosis is based on skin delayed-type hypersensitivity reaction and quantification of interferon gamma but both techniques are influenced by several confounding factors Thus, new methods for early diagnosis are required and miRs have been used as promising biomarker for both infectious and non-infectious diseases. For doing that, we analyzed the expression of four immune-related miRs in 200 cows grouped in cases and controls respect to positivity to tuberculosis. The analysis showed a different magnitude expression in both groups indicating that active tuberculosis could influence miRs expression. Then, we used expression values of miR-146a, the highest differentially expressed miR, for ROC analysis. In order to determine a test cut-off value for miR-146a expression that would differentiate cases and controls, a value for the miR-146a expression higher than 8 was selected as this gave a test specificity and sensitivity of 80.0% and 86.0% respectively. These values confirm the possibility of using miR-146a as milk prognostic biomarker for bovine tuberculosis.

Keywords: microRNAs, biomarker, tuberculosis, case-control study, dairy cows

Bovine tuberculosis (bTB) is a zoonotic disease caused by *Mycobacterium bovis*, a bacteria belonging to the Mycobacterium tuberculosis complex. Disease development is influenced by host genetic background (Papaianni *et al*, 2017) and by a proper immune response. bTB significantly affects animal health influencing herd economic sustainability due to reduction in productivity in severely affected animals. Thus, control and eradication of disease is principally based on removal and slaughter of infected animals following early diagnosis of disease (Vordermeier *et al*, 2016). Diagnosis is based on skin test where the intradermal injection of crude preparation from bacteria gives a delayed-type hypersensitivity reaction in positive animals. This technique is often associated with quantification of interferon gamma in ex-vivo stimulated cell of tested animals. However, these methods are influenced by several confounding factors (Pai *et al*, 2004) and therefore new potential biomarkers are required. microRNAs (miRs) are small non-coding RNA that influence gene expression controlling mRNA degradation (Bartel, 2004). Differential expression of miRs is been often associated with non-infectious (Jeffrey S.S., 2008) and infectious diseases like TB (Vegh *et al*, 2013). Moreover, recent studies have shown that extracellular miRs are stably recovered from body fluid including milk (Weber, 2010) where they have been associated with various biological processes, including immune response (Kosaka *et al*, 2010). Based on these studies, we have measured the expression of some miRs from milk of active infected cows compare with healthy ones. Finally, we have evaluated the potential use of miR-146a as prognostic biomarker for diagnosis of tuberculosis.

Materials and methods

Samples collection

Animals included in the study were from 3 different herds declared infected and were enrolled already in our previous study. As described, they were grouped in cases and controls according to reactivity to resuscitation factor B (Capparelli *et al*, 2013). Milk samples were stored at 4°C and immediately processed once arrived to the laboratory. Then, samples were centrifuged at 3,000 × g for 15 min at room temperature. The supernatant was recovered and further centrifuged at 15,000 × g for 15 min at 4°C. The milk whey was recovered and stored at −80°C for RNA extraction.

RNA Extraction

Before proceeding with RNA extraction from whey milk, 5 fmol of exogenous cel-miR-39 were spiked-in. RNA was isolated using Trizol LS (Thermofisher) following manufacturer's instruction and quantified using NanoDrop. Only samples with both 260/280 and 260/230 ratios >1.8 were used for further investigation.

Real-Time Reverse Transcriptase Quantitative PCR

One microgram of total RNA was reverse transcribed using TaqMan™ MicroRNA Reverse Transcription Kit (Thermo FisherScientific) according to the manufacturer's protocol. Quantitative PCR was performed using a TaqMan Fast Advanced Master Mix kit and a StepOne Plus Real-Time PCR system (Thermo Fisher Scientific). The TaqMan MicroRNA Assays used in this study and their Taqman assay IDs are as follows: bta-miR-146a (ID: 005896), bta-miR-29c (ID: 000587), bta-miR-155 (ID: 002623) and bta-miR99b (ID: 000436) . Thermal cycling was conducted according to the manufacturer's recommended program and using cel-miR-39 as internal control for relative quantification.

Statistical Analysis

All of the milk samples were taken from Holstein-Friesian cows. To reduce potential confounders, all subjects were lactating cow between 40 and 90 month of age and a screening for mastitis was performed immediately in the field using a California mastitis test with a commercial tester for exclude mastitis coinfection. To curb quarter bias, quarter samples were mixed. Data analysis and Receiving operating curve (ROC) were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). Data were analyzed using a parametric unpaired t test, and $P < 0.05$ was considered statistically significant.

Results and discussion

We compared miRs expression in 200 milk samples of Holstein-Friesian cows. Samples were previously grouped in cases (100 animals actively infected) and controls (100 animals negative) according to our previous study (Capparelli *et al*, 2013). In detail, bta-miR-146a, bta-miR-29, bta-miR-155, bta-miR99b were chosen according with previous study where was shown they were differentially regulated in bovine during infection with *M.bovis* [6]. Using a qRT-PCR, we analyzed the expression of the above miRs and all of them were significantly up-regulated in cases versus controls (Table 1). In detail, miR-146a shows the highest difference between cases and controls and the expression data were subjected to ROC analysis to determine if miR-146a could serve as a sensitive and specific prognostic marker identifying animals with active TB. This analysis show that miR-146a could discriminate significantly between cases and controls animals (area under the curve: 0.795 [95% CI: 0.7290 to 0.8614, $p<0.001$] (Figure 1). In order to determine a test cut-off value for miR-146a expression that would differentiate cases and controls, a value for the miR-146a expression higher than 8 was selected as this gave a test specificity of 80.0% (i.e. 20/100 detection of false positive miR-146s responses in control animals). Using this cut-off,miR-146a positive responses were identified in 16/100 controls, giving a test sensitivity of 86.0%. The prognostic accuracy of miR-146a to distinguish cases from controls was also highlighted by calculating the odds ratio after applying the cut-off value described above. Thus, the diagnostic odds ratio was established as being 24, indicating that for an animal showing significant induction of miR-146a in milk the probability of it developing tuberculosis is 24-fold higher than not having it.

Conclusion

This is the first study to demonstrate that miRs, named bta-miR-146a, bta-miR-29, bta-miR-155 and bta-miR99b are differentially regulated in milk from tuberculosis affected dairy cows. This result shows the potential of miRNA in milk for use as a biomarker for bovine tuberculosis. In particular,

we have shown that miR-146a can be used as potential biomarker candidate active tuberculosis infection in cows with 80.0% and 86.0% of specificity and sensitivity respectively.

Acknowledgements

Dr. Marco Iannaccone was supported by research funding from “Fondazione con il Sud” (Project no.2011-PDR-18, “Biosensori piezoelettrici a risposta in tempo reale per applicazioni ambientali e agro-alimentari”)

References

Bartel DP 2004 MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116** 281–297.

Capparelli R, De Chiara F, Nocerino N, Medaglia C, Di Costanzo R, Ramunno L, Capuano F, Casalinuovo F, Di Matteo A & Iannelli D 2013 Heterozygosity at the A625C polymorphic site of the MyD88 gene is associated with Mycobacterium bovis infection in cattle. *Infection and Immunity* **81** 2139-2144.

Jeffrey SS 2008 Cancer biomarker profiling with microRNAs. *Nature Biotechnology* **26** 400–401.

Kosaka N, Izumi H, Sekine K & Ochiya T 2010 microRNA as a new immune-regulatory agent in breast milk. *Silence* **1** 7.

Pai M, Riley LW & Colford JM Jr 2004 Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. *Lancet Infectious Diseases* **4** 761-76.

Papaianni M, Cosenza G, Borriello G, Galiero G, Grasso F, Della Ventura B, Iannaccone M & Capparelli R 2017 The tumor necrosis factor g1022G>A polymorphism is associated with resistance to tuberculosis in water buffalo (Bubalus bubalis). *Animal Genetics* **48** 250-251.

Vegh P, Magee DA, Nalpas NC, Bryan K, McCabe MS, Browne JA, Conlon KM, Gordon SV, Bradley DG, MacHugh DE &. Lynn DJ 2013 Veterinary Immunology and Immunopathology. 55:238-244

Vordermeier HM, Jones GJ, Buddle BM, Hewinson RG, & Villarreal-Ramos B 2016 Bovine Tuberculosis in Cattle: Vaccines, DIVA Tests, and Host Biomarker Discovery. Annual Review of Animal Bioscience 4:87–109.

Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, & Wang K 2010. The microRNA spectrum in 12 body fluids. Clinical Chemestry. 56 1733–1741

	Cases (100)	Control(100)	P-value[a]
bta-miR-146a	9.19 ± 2.83	6.81 ± 1.76	<0.001
bta-miR-29c	6.78 ± 1.22	5.24 ± 1.67	<0.001
bta-miR-155	4.25 ± 1.22	3.44 ± 1.01	<0.001
bta-miR-99b	5.74 ± 1.32	4.56 ± 0.87	<0.001

Table 1. Relative microRNAs expression in milk from both active TB (cases) and control (negative) cows.

[a] Student's t-test.

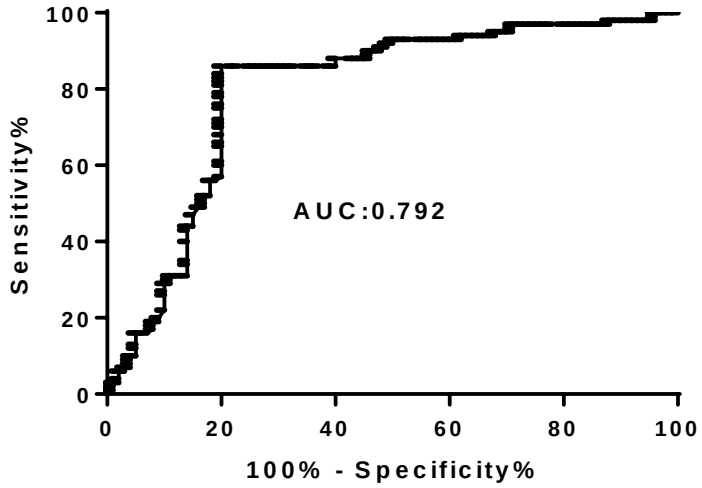

Figure 1. miR-146a as a biomarker of active tuberculosis in milk. Receiver operating characteristic (ROC) analysis to assess efficacy of miR-146a as a biomarker of tuberculosis. AUC,area under the curve.