Original Article

J Babol Univ Med Sci
Vol 18, Issu 7; Jul 2016. P:19-23

Evaluation of Antimicrobial Activity of Lactoferrin against P.Aeruginosa and E.Coli Growth

R. Sharbafi (MSc)[1], F. Moradian (PhD)*[2], A. Rafeiei (PhD)[1]

1.Molecular and Cellular Biology Research Center, Faculty of Medicine, Mazandaran University of Medical Science, Sari, I.R.Iran
2.Department of Basic Sciences, Sari Agricultural Sciences and Natural Resources University, Sari, I.R.Iran

J Babol Univ Med Sci; 18(7); Jul 2016; PP: 19-23
Received: Feb 13th 2016, Revised: Mar 2th 2016, Accepted: Jun 1th 2016

ABSTRACT

BACKGROUND AND OBJECTIVE: Lactoferrin(LF) is an iron-binding glycoprotein that involves a diverse range of biological activities. Lactoferrin is a major component of milk and is present in exocrine secretions such as tears, salvia, bile, and neutrophil granules. Lactoferrin has more potent antimicrobial activities against a wide range of gram negative and positive bacteria as well as antivirus activities. The purpose of this study is to evaluate the effect of this protein on P.aeruginosa growth in patients with burns that show drug resistance.

METHODS: In this study, antibacterial activity of Lactoferrin has been scrutinized after isolation and purification of bovine colostrum against pseudomonas aeroginosa. Bacteria samples were isolated from scald patients (Shahid Zare Hospital); then microbial activity was confirmed with biochemical tests like oxidase, catalase and growth on TSI medium. Four concentrations 400,500,600 and 700 µg/ml of lactoferrin were assayed. Pseudomonas colonies counted and compared with negative control (without lactoferrin) as well as *E.coli* (DH5α) as positive control was considered.

FINDINGS: Our results showed that 400µg/ml concentration of lactoferrin has the least inhibitory effect with 35% and 29% growth inhibitory and 700µg/ml concentration of lactoferrin has the highest inhibitory effect with 86% and 66% on *Pseudomonas* and *E.coli*, respectively.

CONCLUSION: Our result showed that all of lactoferrin concentrations have inhibitory activity which in 700µg/ml has the highest inhibition against Pseudomonas aeroginosa and also E.coli.

KEY WORDS: *Antimicrobial activity, E.coli, lactoferrin, Pseudomonas aeroginosa*.

Please cite this article as follows:
Sharbafi R, Moradian F, Rafeiei A. Evaluation of Antimicrobial Activity of Lactoferrin against P.Aeruginosa and E.Coli Growth. J Babol Univ Med Sci. 2016;18(7):19-23.

***Corresponding author: F. Moradian (PhD)**
Address: Department of Basic Sciences, Sari Agricultural Sciences and Natural Resources University, Sari, I.R.Iran
Tel: +98 11 32687655
E-mail: moradi_f@yahoo.com

[DOR: 20.1001.1.15614107.1395.18.7.3.9]
[DOI: 10.22088/jbums.18.7.19]

Introduction

Lactoferrin is an 80-kDa glycoprotein that has the ability of binding to iron. This protein can be found in milk, tear, saliva and specific granules (PMNS), particularly neutrophils. Its biological properties include regulation of intestinal iron absorption, anti-inflammatory properties, regulation of the immune system and anti-bacterial, anti-viral and anti-tumor activities (1).

The effect of lactoferrin in reducing inflammation and destroying gram-negative bacteria is followed by clinical outcomes such as infection control, multiple organ dysfunctions and bacterial invasion (2). The protein structure consists of two globular lobes, designated as N-lobe and C-lobe, each of which contains iron-binding site (3, 4).

In some cases, lactoferrin binds to iron released from transferrin that prevents the proliferation of bacteria (5). Lactoferrin's tendency to absorb iron is considered as the main composition in non-specific immune system of host against numerous pathogens (6). By absorbing free iron, lactoferrin clears the environment of this element, thus bacteria and pathogens which need iron to grow and proliferate will be deprived of this element and cannot survive and therefore lactoferrin inhibits the infection indirectly (7). In addition to absorbing iron and keeping it away from microbes, lactoferrin can have antimicrobial activity by inhibiting microbial metabolism of carbohydrates, destabilizing bacterial cell membrane and binding to calcium and magnesium (8). Using lactoferrin in feeding of animals such as mouse and pig has increased their resistance to bacterial and inflammatory diseases (9).

Pseudomonas consists of various species of non-fermentative aerobic gram-negative bacilli in soil and water which are scattered widely in nature, water, sewage, dust, air and soil (10). One of the most common nosocomial infections created by an opportunistic pathogen such as pseudomonas aeruginosa due to immunodeficiency is post-burn infection. Since resistance to antibiotics is a major challenge in treating these infections, finding an effective alternative treatment is a medical priority. According to the long history of lactoferrin's benefits in treating various diseases and the effect of this edible protein in strengthening immune system to fight against various pathogens including bacteria, antibacterial effect of lactoferrin purified from bovine colostrums at various concentrations on pseudomonas collected from samples of burn patients was investigated in this study.

Methods

Sampling, isolation and identification of bacteria: Samples were collected from the wound site of burn patients (Shahid Zare hospital in Sari) with sterile swab and were transferred to blood agar plates. Plates were incubated at 37ºC for 24 hours. In order to detect bacteria, biochemical tests such as urease, oxidase, catalase, indole and citrate were performed according to standard methods. Moreover, samples were cultured in Kligler Iron Agar (KIA) and Triple Sugar Iron (TSI) medium (11).

Extraction, purification and determining the concentration of lactoferrin: lactoferrin was isolated from bovine colostrums within 72 hours after delivery and was purified with ion exchange chromatography (CM Sephadex® C-50); its purity was confirmed with SDS-Page gel and its concentration was measured with Bradford assay using bovine serum albumin (BSA) as standard. For this purpose, different concentrations of the standard were prepared first, then two different volumes of lactoferrin were prepared and equal amount of Bradford reagent was added to all tubes. Absorbance of the samples at 595 nm was read and the concentration of purified lactoferrin in accordance with the standard curve plotted for serum albumin protein was 2.5 µg/µl. The result of purification has been reported in previous studies by Sharbafi et al. (12).

Microbial culture and investigating the antimicrobial activity of lactoferrin: *Pseudomonas* was first cultured in 50 ml LB broth medium for 18 hours and absorption of (OD) broth was read with spectrophotometer after this period. Absorption of broth after 18 hours of incubation was 0.2 which is equal to 3 McFarland standard. Then 12 dilutions from 10^{-1} to 10^{-12} were prepared from primary broth medium and absorption of each dilution was red separately. 100 µl of each dilution was cultured in plates containing eosin methylene blue (EMB).

After incubation for 18 hours at 37ºC, grown colonies were counted. Dilution of 10^{-12} with OD had 0.2 countable colonies; this dilution showed the most appropriate number of countable colonies. 5 plates containing eosin methylene blue (EMB) was prepared and 100 µl of 10^{-12} dilution was added to each plate, then 4 different concentrations of lactoferrin including 400, 500, 600 and 700 µg/ml were added to 4 plates

[DOR: 20.1001.1.15614107.1395.18.7.3.9]
[DOI: 10.22088/jbums.18.7.19]

and one plate without lactoferrin (as negative control) was considered. The plates were incubated at 37ºC for 18 hours. The same procedure was performed on *Escherichia coli* strain as a positive control. After incubation and growth of bacteria, colonies were counted and the level of colony forming unit (CFU) was specified.

Statistical analysis: This study was conducted in a completely randomized design and after ensuring normality of data with One Sample Kolmogorov-Smirnov Test, data analysis of the effect of different concentrations of lactoferrin on bacterial growth was done using SPSS 19 software, one-way analysis of variance (one-way ANOVA) and Duncan's multiple range test (MRT) among different treatments and $p<0.05$ was considered significant.

Results

Results of this study demonstrated that lactoferrin actively reduces the growth of *Pseudomonas aeruginosa* and the percentage of inhibition was 35, 58, 75 and 86, respectively; minimum inhibitory concentration (MIC) pertained to concentration of 400 µg/ml with 35% inhibition and maximum percentage of inhibitory activity of lactoferrin pertained to concentration of 700 µg/ml with 86% inhibition of pseudomonas aeruginosa (Fig 1).

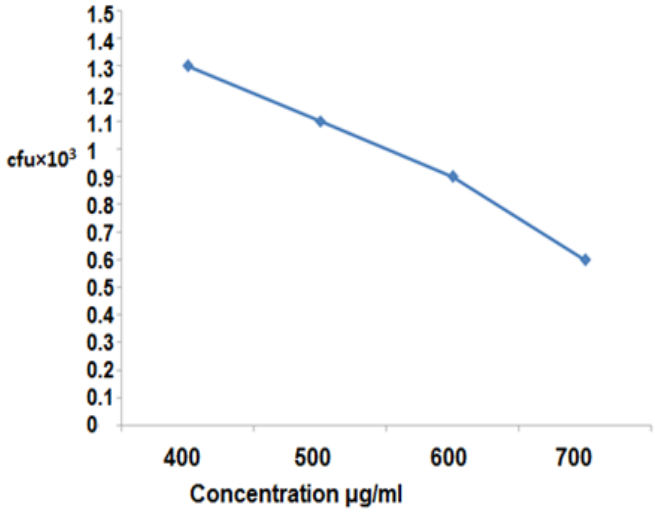

Figure 1. The effect of different concentrations of lactoferrin on *Pseudomonas aeruginosa* growth

Lactoferrin decreased the number of colonies in *Escherichia coli* too and the percentage of inhibition was 29, 37, 52 and 66, respectively; minimum inhibitory concentration (MIC) pertained to concentration of 400 µg/ml with 29% inhibition and maximum percentage of inhibitory activity of lactoferrin pertained to concentration of 700 µg/ml with 66% inhibition of *Escherichia coli* (Fig 2). These results demonstrated that inhibitory effect of lactoferrin on Pseudomonas aeruginosa is more than its effect on *Escherichia coli* as a positive control (Fig 3).

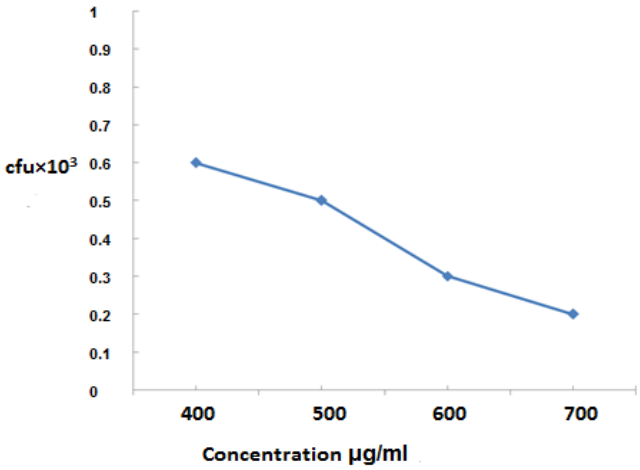

Figure 2. The effect of different concentrations of lactoferrin on escherichia coli growth

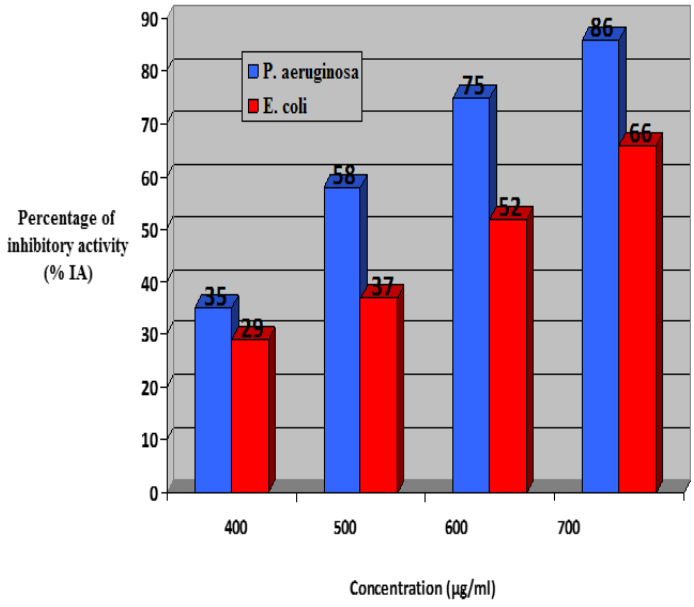

Figure 3. Comparing percentage of lactoferrin's inhibitory activity against P. aeruginosa and E. coli

Discussion

Lactoferrin is a multifunctional protein with antibacterial properties that affects a wide range of

[DOR: 20.1001.1.15614107.1395.18.7.3.9]
[DOI: 10.22088/jbums.18.7.19]

gram-negative and gram-positive species. Difference between our results and previous studies is related to the level of inhibitory concentration. Woan-sub et al. have reported antimicrobial activity of lactoferrin against various species of *Pseudomonas, Pseudomonas syringae* and *Pseudomonas fluorescens*, while highest inhibitory effect of lactoferrin pertained to concentration of 1.9 mg/ml. They also reported that *Pseudomonas syringae* showed more sensitivity towards lactoferrin compared with *Pseudomonas fluorescens* (13).

Takase et al. have studied the antimicrobial effect of lactoferrin on *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains isolated from the udder. In this experiment the concentration of 0.67, 1.67and 2.67 mg/ml was used. Their results have demonstrated that concentration of 2.67 mg/ml has the highest inhibitory effect and lactoferrin is most effective for inhibition of *Escherichia coli* and *Pseudomonas aeruginosa* growth. These scientists have also indicated that lactoferrin inhibits their growth by damaging the gram-negative outer membrane and altering bacterial outer membrane permeability (14). Dionysius et al. have reported that lactoferrin with concentration of 1 mg/ml inhibits the growth of ETEC (enterotoxigenic *Escherichia coli*) separated from patients with inflammatory bowel disease. They have indicated that inhibitory effect of lactoferrin depends on the type of strain and initial number of bacteria (15). Antimicrobial activity of lactoferrin against various species of *Pseudomonas* can be described with a number of mechanisms. The first mechanism is that lactoferrin is an iron-binding protein. Iron is a vital element for every microorganism. Decrease in concentration of free iron in the environment of microorganism creates a situation that eventually leads to their death. On the other hand, lack of iron in the environment prevents *Pseudomonas aeruginosa* from generating biofilm. The second mechanism indicates that lactoferrin disrupts the membrane of gram-negative bacteria by binding to lipid A. Destabilization of bacterial outer membrane releases lipopolysaccharides (LPS) and eventually alters bacterial outer membrane permeability. Altering bacterial outer membrane permeability supplements the antimicrobial activity of lactoferrin by allowing entrance of antibiotics and other antimicrobial agents (16).

Results of the present study demonstrated that lactoferrin decreases *Pseudomonas aeruginosa* growth effectively in vitro. Inhibitory effect on *Escherichia coli* and *Pseudomonas* strains was witnessed in lower concentrations of lactoferrin compared with previous studies, suggesting that the obtained lactoferrin is more pure and active and that the strains tested in this study are more sensitive to lactoferrin.

Acknowledgments

Hereby, we would like to thank the Tabarestan Institute of Genetics and Biotechnology (Sari) and Molecular and Cell Biology Research Center of Mazandaran University of Medical Sciences for their unsparing cooperation

[DOR: 20.1001.1.15614107.1395.18.7.3.9]
[DOI: 10.22088/jbums.18.7.19]

References

1.Abrink M, Larsson E, Gobl A, Hellman L. Expression of lactoferrin in the kidney: implications for innate immunity and iron metabolism. Kidney Int. 2000;57(5):2004-10.

2.Drago-Serrano ME, de la Garza-Amaya M, Luna JS, Campos-Rodríguez R. Lactoferrin-lipopolysaccharide (LPS) binding as key to antibacterial and antiendotoxic effects. Int Immunopharmacol. 2012;12(1):1-9.

3.Metz-Boutigue MH, Jollès J, Mazurier J, Schoentgen F, Legrand D, Spik G, et al. Human lactotransferrin: amino aeid sequence and structural comparisons with other transferring. Eur J Biochem. 1984;145(3):659-76.

4.Baker EN. Structure and reactivity of transferrins. Adv Inorg Chem. 1994;41:389-463.

5.Valenti P, Antonini G. Lactoferrin: an important host defense against microbial and viral attack. Cell Mol Life Sci. 2005; 62(2): 2576-87.

6.Velusamy SK, Poojary R, Ardeshna R, Alabdulmohsen W, Fine DH, Velliyagounder K. Protective effects of human lactoferrin during aggregatibacter actinomycetemcomitance induced bacteremia in lactoferrin efficient mice. Antimicrob Agents Chemother. 2014; 58(1):397-404.

7.Crosa J.H. Genetics and molecular biology of siderophore-mediate iron transport in bacteria. Microbiol Rev. 1989;53(4):517-30.

8.Appelmelk BJ, An YQ, Geerts M, Thijs BG, de Boer HA, MacLaren DM, et al. Lactoferrin is a lipid A- binding protein. Infect Immun. Infect Immun. 1994;62(6):2628-32.

9.Barker EN, Barker HM. Molecular structure, binding properties and dynamics of lactoferrin.Cell Mol life Sci. 2005; 62(22):2531-25 39.

10.Rahimi MK. Zinser Microbiology. Tehran: Abizh Pub; 1382.p.2.[In Persian]

11.Sneath PHA. Endospore–forming gram–positive rode and cocci. In: Bergey's manual of systematic bacteriology. USA: Williams and Wikins, Baltimore; 1983.p.1104-207.

12.Sharbafi R, Moradian F, Rafiei F, Barzegar A. Isolation and purification of bovine lactoferrin. J Mazandaran Univ Med Sci. 2011; 21(84): 44-51. [In Persian]

13.Woan-sub Kim, Rahman MM, Kei-Ichi Shiazaki I. Antibacterial activity and binding ability of bovine lactoferrin against pseudomonas Spp. J Food Safety. 2008; 28(1):23-33.

14.Takase K, Hagiwara K, Onodera H, Nishizawa Y, Ugaki M, Omura T, et al. Constitutive expression of human lactoferrin and its N-lobe in rice plants to confer disease resistance. Biochem Cell Biol. 2005;83(2):239-49.

15.Dionysius DA, Grieve PA, Milne JM. Forms of lactoferrin: Their Antibacterial effect on Enterotoxigenic Escherichia coli. J Dairy Sci. 1993;76(9):2597-600.

16.Brink W. Lactoferrin:the bioactive peptide that fights disease. Life Exten Mag. 2000. Available from: http://www.lifeextension.com/magazine/2000/10/report_lactoferrin/page-01

[DOR: 20.1001.1.15614107.1395.18.7.3.9]
[DOI: 10.22088/jbums.18.7.19]