Involvement of host iron-withholding strategy on *Streptococcus pyogenes* strain KSU-1 growth and pathogenicity

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Background and objectives
Streptococcus pyogenes is a highly adaptable human pathogen that can cause a wide spectrum of infections ranging from mild to a life-threating systemic infection. This study discussed the effectiveness of iron-depriving strategy on growth, survival, and virulence of *S. pyogenes*.
Materials and methods
Some comparisons between different iron-saturated and iron-depleted forms of the main human iron reservoirs (hemoglobin, hemin, transferrin, lactoferrin, and human milk) were tested for their effect on growth and pathogenicity of *S. pyogenes*.
Results and conclusion
Although the iron-saturated forms enhanced the growth and survival, the iron-free forms had a bacteriostatic/bactericidal activity against the microbe, and these results were emphasized by the *in vivo* study. Finally, the bacterial surface proteins as virulence factors were secreted upon iron depletion as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. This study proved that iron-depletion environment increased the resistance and virulence factors of *S. pyogenes*. Moreover, these results can give an insight about the interaction between the host and pathogen, which can lead to designing of new antimicrobial agents or vaccine that may target these pathways.

Keywords:
iron restrictions, lactoferrin, pathogenicity, *Streptococcus pyogenes*, transferrin

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Introduction

Streptococcus pyogenes, a beta-hemolytic bacterium that belongs to Lancefield serogroup A, also known as the group A streptococci (GAS), causes a wide variety of diseases in humans. Infection with GAS comprises a public health problem, causing mild to severe illness, and its repeated infections may cause several autoimmune diseases [1]. The virulence of this organism is related to its adaptation to human host and its ability of adhesion and colonization to host surface. The mechanisms of adhesion–receptor interaction existing between the pathogen and human matrix molecules include different substrates such as collagen, fibronectin, and laminin that can be used by the pathogen [2]. Fibronectin also was found in the blood plasma and amniotic fluids, so it has the main role in cell growth, adhesion, differentiation, wound healing, and interaction with pathogens. *S. pyogenes* can express about 12 distinct fibronectin-binding proteins [2,3].

It was proved that the growth and virulence of some *Streptococci* species was affected by their ability to recognize host receptors and uptake host iron in the form of protein-containing iron or heme [4]. For example, host iron can be stored intracellularly as ferritin, or extracellular as transferrin (TF) and lactoferrin (LF), and also, hemoglobin (Hb) was proved to have a vital role during pathogenicity [5,6]. GAS can use successfully Hb, heme–albumin, myoglobin, and catalase as sources of iron during its growth in laboratory media, but it cannot use TF or LF [7] because LF is one of the TF proteins that has antimicrobial activity against a broad spectrum of pathogens and biofilms [8]. Therefore, to combat GAS infections, its mode of action in the initiation of infection must be investigated and understood. Therefore, the critical point here is the detection of iron competition existing between the host and the pathogen. Throughout the phase of infection, an important strategy for defense by the host is the suppression of available iron levels in serum and secretions to enhance host natural resistance on one hand and to affect the bacterial growth on the other hand [9]. In this case, owing to the importance of iron

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for bacterial growth because of its involvement in some bacterial essential enzymes [10], bacteria use some mechanisms to uptake iron from the host such as iron acquisition [11] or uptake [7] and the multimetal transporter [12]. Thus, iron level became a critical factor for the formation of bacterial virulence factors [12–14]. Moreover, this represents important consequences not only for understanding bacterial pathogenicity but also for the design of potential measures applied to prevent or treat infections in addition to identification of the potential targets for novel antibiotic treatments or vaccines [4].

This study aimed to determine the effect of iron starvation (*in vitro* and *in vivo*) on growth of *S. pyogenes strain KSU-1* and related protein profiles. This leads to understand the mechanism of iron uptake and detect its role in bacterial virulence to provide new ways for treatment of bacterial infections.

Materials and methods

Microorganism

S. pyogenes isolates were obtained from the Microbiology Laboratory Military Hospital (Riyadh, Saudi Arabia). The bacterial strains were grown in brain heart infusion (BHI) agar media (Oxoid) for 24 h at 37°C. After the incubation period, single colonies were picked and subcultured several times in fresh BHI agar plates to obtain homogeneous pure culture. Then, the isolates were grown overnight in BHI broth at 37°C, and bacterial glycerol stock was prepared using sterilized glycerol solution (20%) and stored at −80°C.

Bacterial identification

Five *S. pyogenes* suspected isolates, termed KSU-1 to KSU-5 strains, were plated onto blood agar (BA) with defibrinated sheep blood (5%) and incubated for 24–48 h in a CO_2 incubator at 37°C. The isolate was identified according to colonial morphology, Gram's staining, beta hemolysis, conventional biochemical tests, bacitracin sensitivity, and positive for Lancefield group A antigen. The isolate was further identified using API20 kits (BioMerieux Inc., Lyon, France). In brief, individual colony of the bacterial isolate was subcultured on BHI agar plates and incubated at 37°C for 24 h. After the incubation period, the cells were harvested using a sterile loop and suspended in a sterile saline solution to be equivalent to 0.5 McFarland standards. This bacterial suspension was used to inoculate the API20 strips, and the results were interpreted according the manufacturer's instructions [15].

Culture conditions and inoculum preparation

The selected *S. pyogenes* strain was grown in BHI agar media Oxoid (Hampshire, UK) for 24 h at 37°C. For culture inoculum preparation, the strain was grown on BHI broth at 37°C for 18 h at 120 rpm, and then, the cells were harvested and washed twice with deionized water. After that, the cell pellet was resuspended in sterile saline to the desired cell concentration (10^6 CFU/ml of saline), which was used as inoculum in the next experiments.

Effect of iron starvation on *Streptococcus pyogenes*

Iron starvation using $MgCO_3$

BHI broth was prepared and depleted of iron by adding $MgCO_3$ as an iron chelator at a concentration of 80 mg/ml. Then, iron was precipitated from the BHI broth with continuous stirring followed by centrifugation at 9000 rpm for 20 min. The supernatant was sterilized by autoclaving and used as an iron-free medium [16]. After that, the bacterial suspension was inoculated in the iron-depleted and complete BHI medium and incubated 24 h at 37°C in a shaker adjusted at 120 rpm. The bacterial growth was measured at zero time and then at 3-h intervals for a period of 12 h at 660 nm. BHI broth without any addition was used as a control. All experiments were carried out in triplicate, and the mean values were reported.

Iron starvation using ethylenediamine-di-o-hydroxyphenyl acetic acid

The iron chelator ethylenediamine-di-o-hydroxyphenyl acetic acid (EDDA) was purchased from Winlab Ltd (Watford, England). First, EDDA was treated to remove any possible iron traces in its composition as follows [17]: a solution containing 10 g EDDA in 90 ml of boiling 1 N HCl was prepared. Then after being cooled and filtered, it was diluted with 1500 ml of acetone, and the pH was raised to 6.0 by adding 1 M of NaOH. After standing overnight at 4°C, the EDDA precipitate was collected by filtration and washed with cold acetone. Thereafter, the deferrated EDDA was added to flasks containing 50 ml of BHI broth, at final concentration of 170 μg/ml, and completely dissolved in the medium before sterilization. The flasks were then inoculated with saline-washed bacterial cells to give a final optical density of 0.1 at 660 nm and then incubated overnight at 37°C in a shaking incubator (120 rpm). The bacterial growth (OD 660 nm) was measured at zero time and then at 3-h intervals up to 12 h.

Effect of iron-free and iron-saturated host-binding proteins on *Streptococcus pyogenes*

Preparation of lactoferrin, transferrin, hemoglobin, and hemin

Human LF, TF, Hb, and hemin were purchased from Sigma Chemical Company (NY, USA). The iron-free

forms of LF, TF, Hb, and hemin were prepared by dialysis against solution containing 0.2 M sodium acetate, 0.2 M NaH_2PO_4, and 0.4 M EDTA (pH 4.0) for 16 h with stirring at 4°C and changing solution every 4 h. Then, the dialysis bags were transferred into deionized water for further dialysis for 4 h before use. On the contrary, the iron-saturated LF, TF, Hb, and hemin were prepared by dialysis against 0.1 M ferrous ammonium sulfate. All preparations were filtered using a sterilized 0.45 μm Millipore filter [18].

Effect of transferrin, hemoglobin, and hemin on Streptococcus pyogenes

Sterilized iron-free TF, Hb, and hemin and iron-saturated TF, Hb, and hemin were added to 50 ml of sterilized BHI broth giving a final concentration of 150 μg/ml of each one. Thereafter, the flasks were inoculated with saline-washed bacterial cells and then incubated overnight at 37°C in a shaking incubator adjusted at 120 rpm. The bacterial growth optical density was measured at zero time and then at 3-h intervals for a period of 12 h at 660 nm. BHI broth without any addition was used as a control [16].

Effect of pure lactoferrin and human milk on Streptococcus pyogenes

Samples of human milk colostrum (obtained from healthy mothers, King Khalid University Hospital, Riyadh, Saudi Arabia) were centrifuged at 8000 rpm and then iron-free and iron-saturated human milks were prepared as described before. Then, 2 ml of iron-saturated and iron-free LF (1.7 mg/ml) and 2 ml of iron-free and iron-saturated human milk were added to separate flasks containing 13 ml of BHI broth. All flasks were then inoculated with saline-washed cells of *S. pyogenes* to give a starting optical density of 0.1 at 660 nm [16]. The optical density was measured at zero time and then at 3 h.

Bactericidal activity of human milk

For investigation of the bactericidal activity of human milk against *S. pyogenes,* samples of iron-free and iron-saturated human milk were added to *S. pyogenes* cell suspension (200 μl human milk/10^6 cells in 1 ml of deionized water) and incubated at 37°C. The control was prepared by addition of similar volume of sterilized deionized water instead of human milk to the cell suspension. Thereafter, cell viability was tested by withdrawing samples after specific time intervals. Samples were washed by sterile deionized water and centrifugation at 5000 rpm, resuspended in sterile deionized water, and serially diluted. Then, the samples were spread on BA plates and incubated at 37°C for 4 h, and the obtained colonies were counted.

Reversible and irreversible inhibition activity of iron-free lactoferrin/or transferrin

Sterile iron-free LF and TF were added to 15 ml of BHI broth (1 mg/ml, pH 7.2) in separate flasks. BHI broth without LF or TF was used as the control. All flasks were inoculated with 10^4 cells/ ml and incubated at 37°C, 120 rpm. Samples were removed at zero time and then at 4-h intervals, centrifuged, diluted with a sterile saline, and spread on BA plates, and then the obtained colonies were counted after 48 h of incubation at 37°C. The reversibility of inhibition was tested after 8 and 12 h by adding iron-saturated LF or iron-saturated TF (3 mg/ml) to the appropriate flasks. On the contrary, irreversible inhibition was done by applying the same experiment but the cells were suspended first in deionized water and then both iron-free LF and TF were added to the same concentration of the medium and compared with the control. After incubation at 37°C, 120 rpm for 12 h, the cells were centrifuged and resuspended in iron-complete BHI broth and incubated at the same conditions. Samples were removed at zero time and then at 4-h intervals up to 20 h, diluted, plated on BA plates, and counted after 48 h incubation at 37°C.

In vivo effect of lactoferrin on survival of Streptococcus pyogenes

Swiss–Webster mice (6–8 weeks old) were obtained from the Experimental Animal Center, College of Pharmacy, King Saud University. The mice were injected intraperitoneally with $FeSO_4.7H_2O$ (40 μg), iron-saturated LF, and iron-free LF (3 mg), EDDA (2.5 mg) followed by injection of *S. pyogenes* suspensions after 50 min. In addition, to investigate the effect of excess iron/EDDA ratio, another group of mice were injected with excess $FeSO_4$ (60 μg) and less EDDA (1.25 mg) mixture, 6 h after bacterial injection. The control mice were injected with an equivalent volume of saline. For quantitation of bacterial survival, a bacterial suspension (10^6 cells/0.1 ml saline) was injected into the peritoneal cavity. Then, mice were killed at 3-h intervals with CO_2 gas. Six milliliters of normal saline was injected into the peritoneum, and after kneading the abdomen for 1 min with blunt forceps, the fluid was removed, diluted, and spread on BA plates to obtain viable count. All animal experiments followed the ethical standards for experimental animals care and use approved by the Ethical Committee of Animal Care in King Saud University [19].

Isolation of surface proteins of *Streptococcus pyogenes* using sodium dodecyl sulfate polyacrylamide gel electrophoresis

Defferated EDDA (192 µg/ml), iron-free LF (230 µg/ml), and iron-saturated LF (230 µg/ml) were added to separate flasks containing 26 ml of normal BHI broth in addition to iron-depleted BHI broth. All flasks were inoculated by bacterial cells and incubated with shaking (120 rpm) at 37°C for 12 h. The surface proteins of bacterial cells were extracted as described previously by Al-Salamah and Al-Obaidi [16] with some modifications. Cells were harvested by centrifugation at 7000 rpm for 10 min. The pellet was incubated with lysozyme (1 mg/ml) for 30 min and then suspended in 5 ml of 10 mM Tris buffer (pH 7.5) containing 5 mM $MgCl_2$. Thereafter, the cells were sonicated, and the unbroken cells were removed by centrifugation. The supernatant fluid was sedimented by centrifugation at 15 000 rpm for 25 min. The sediment was suspended in 500 µl of 10 mM Tris, 5 mM $MgCl_2$, with 2% Triton X-100, pH 8.0, and incubated at room temperature for 30 min. Samples were precipitated for 30 min and then resuspended in 50 µl of electrophoresis buffer [20]. Equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis in a 12% gel [21].

Statistical analysis

All experiments were carried out in triplicate, and the mean values were reported. All statistical analyses and *P* value calculations were performed with Microsoft Excel 2010.

Results and discussion

Bacterial identification

The *S. pyogenes* strain KSU-1 isolate showed smooth and entire colonies surrounded by defined clear zone owing to the complete degradation of the red blood cells in the BA medium, which indicated that our organism is of beta hemolytic group A streptococcus [22]. This characteristic can be used as an early step in identification of this clinical isolate. Moreover, the isolate was found to be gram-positive cocci arranged in short or long chain or sometimes in clusters, and it was catalase negative. Furthermore, the organism was bacitracin susceptible, so it was from GAS [15]. Furthermore, API20 kits identified the isolates as *S. pyogenes* (n=5). Bacterial identification by API20 Strep was used for streptococci identification, and it is become available for human strains [23]. In that issue, Poutrel and Ryniewicz [24] identified correctly 16 strains using API20 Strep, and they proved that there was 92% accordance with the biochemical identification. *S. pyogenes* strain KSU-1 was used in all of the following experiments.

Effect of iron starvation on *Streptococcus pyogenes* growth

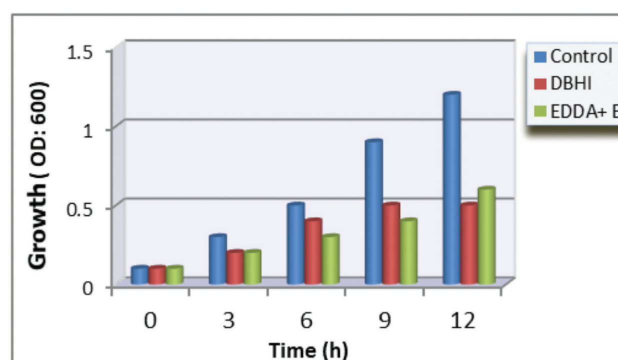
The effect of iron starvation on *S. pyogenes* growth was investigated by removal of iron from the BHI broth medium using two different iron chelators, including $MgCO_3$ and EDDA. As shown in Fig. 1, the *S. pyogenes* strain showed good growth on the normal medium that contains iron, whereas the growth rate was significantly reduced in response to addition of both iron chelators during the log phase. The results indicated that iron is important for optimum growth of *S. pyogenes* owing to its involvement in some essential enzyme and cofactors and for metabolism [10]. Similar results were obtained by previous studies [6,25–27].

Effect of iron-free and iron-saturated host-binding proteins on *Streptococcus pyogenes*

Use of hemoglobin and hemin as a source of iron

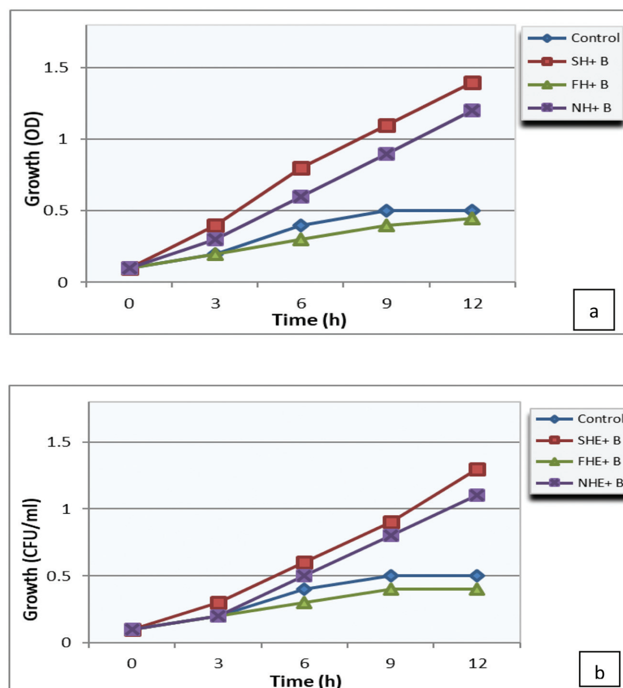
The addition of some iron supplements as Hb or hemin in three different forms (iron-saturated, iron-free, and natural one) compared with control (iron-depleted medium) was investigated (Fig. 2a and b). The results revealed that the growth of *S. pyogenes* was enhanced in the presence of both saturated and natural Hb or hemin but inhibited in the presence of iron-free Hb or hemin. However, Hb is considered as the largest iron reservoir in the body, and at the same time, it is an important iron source for most pathogens, but the full mechanisms of iron acquisition from hemoprotein by *S. pyogenes* are not yet fully

Figure 1



Comparison between *Streptococcus pyogenes* growth rate under normal and iron-limitation conditions. Symbols: control (normal BHI media and bacteria), DBHI (iron-depleted BHI and bacteria), and EDDA+B (EDDA, BHI, and bacteria). All experiments were carried out in triplicate, and mean values were illustrated (P=0.231). The SDs were in the range of 1.8–2.1%. BHI, brain heart infusion; EDDA, ethylenediamine-di-o-hydroxyphenyl acetic acid.

Figure 2



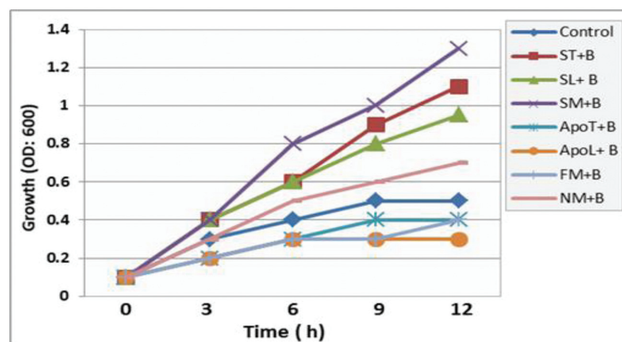
Utilization of hemoglobin (a) and hemin (b) as source of iron by *Streptococcus pyogenes*. Control (iron-depleted BHI, bacteria), SH +B (iron-saturated hemoglobin, DBHI bacteria), SHE+B (iron-saturated hemin, DBHI, bacteria), FH+B (iron-free hemoglobin, DBHI, bacteria), FHE+B (iron-free hemin, DBHI, bacteria), NH+B (natural hemoglobin, DBHI, bacteria), and NHE+B (natural hemin, DBHI, bacteria). All experiments were carried out in triplicate, and the mean values are illustrated (P=0.08). The SDs were in the range of 1.0-–1.4%. BHI, brain heart infusion.

understood [28]. But recently, it is believed that iron uptake from heme during *in vivo* experiments was affected by the production of hemolysins or toxins, which disrupt the cellular membrane to release heme outside the cell [29]. Moreover, Akhter *et al.* [6] found that the pathogen can benefit from Hb during its *in vitro* cultivation; consequently, they concluded that the pathogen can use it during its *in vivo* adaptation, and these new findings can provide good insight about the interactions between the pathogen and human host that can lead to design of potential antimicrobial agents or vaccine that may target these pathways.

Effect of iron-free and iron-saturated forms of lactoferrin, transferrin, and human milk

The growth of *S. pyogenes* was investigated using deferrated BHI broth in the presence of iron free and iron saturated forms of TF, LF, and human milk. The results indicated that the growth of *S. pyogenes* increased in the presence of iron-saturated forms of TF, LF, and human milk, but it was inhibited by iron-free forms of the same tested materials (Fig. 3). This indicated that *S. pyogenes* was able to acquire iron from the iron-saturated bind proteins including human

Figure 3



Effect of TF, LF, and human milk against the growth of *Streptococcus pyogenes*. Symbols: control (DBHI, bacteria), ST+B (iron-saturated TF, DBHI, bacteria), SL+B (iron-saturated LF, DBHI, bacteria), SM+B (iron-saturated human milk, DBHI, bacteria), Apo-T+B (iron free TF, DBHI, bacteria), Apo-L+B (iron-free LF, DBHI, bacteria), FM+B (iron-free human milk, DBHI, bacteria), and NM+B (natural human milk, DBHI, bacteria). All experiments were carried out in triplicate, and the mean values were illustrated (P=0.06). The SDs were in the range of 1.5–3.1%. BHI, brain heart infusion; LF, lactoferrin; TF, transferrin.

milk, LF, and TF, leading to increase in the growth. On the contrary, growth was significantly reduced in the presence of iron-free TF (Apo TF) and iron-free lactoferrin (Apo-LF), which is owing to their high ability to withhold iron from the pathogens because of their high affinity toward iron, thereby exerting a bacteriostatic activity against *S. pyogenes* [10,30] The apparent ability of *S. pyogenes* to acquire iron from human TF and LF suggests that *S. pyogenes* may produce iron chelator called siderophore to uptake iron or it can take it directly from LF or TF, and this siderophore may be exploited by the host cell for growth and iron uptake [31,32].

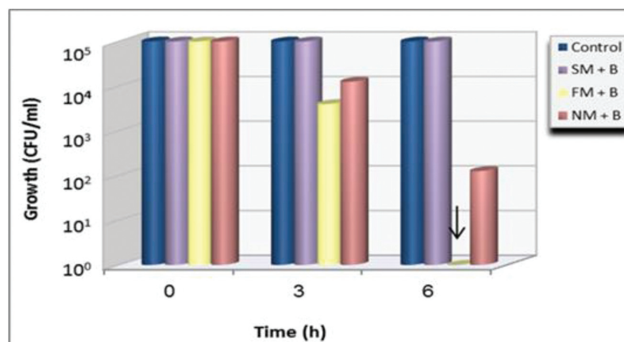
Bactericidal activity of human milk

For investigation of the bactericidal activity of human milk against *S. pyogenes*, samples of iron-free and iron-saturated human milk were added to *S. pyogenes* cell suspension; thereafter, cell viability was tested by withdrawing samples after specific time intervals. The results shown in Fig. 4 indicated that the viability of *S. pyogenes* cells was markedly reduced by incubation with iron-free human milk followed by natural human milk, where the bactericidal activity was completely lost when the milk was saturated with iron. Human milk contains a wide variety of proteins that have an important role as antimicrobial agents and can raise the immune-stimulatory functions [30], proving the previous experiment results.

Reversible and irreversible inhibition by iron-free lactoferrin and transferrin

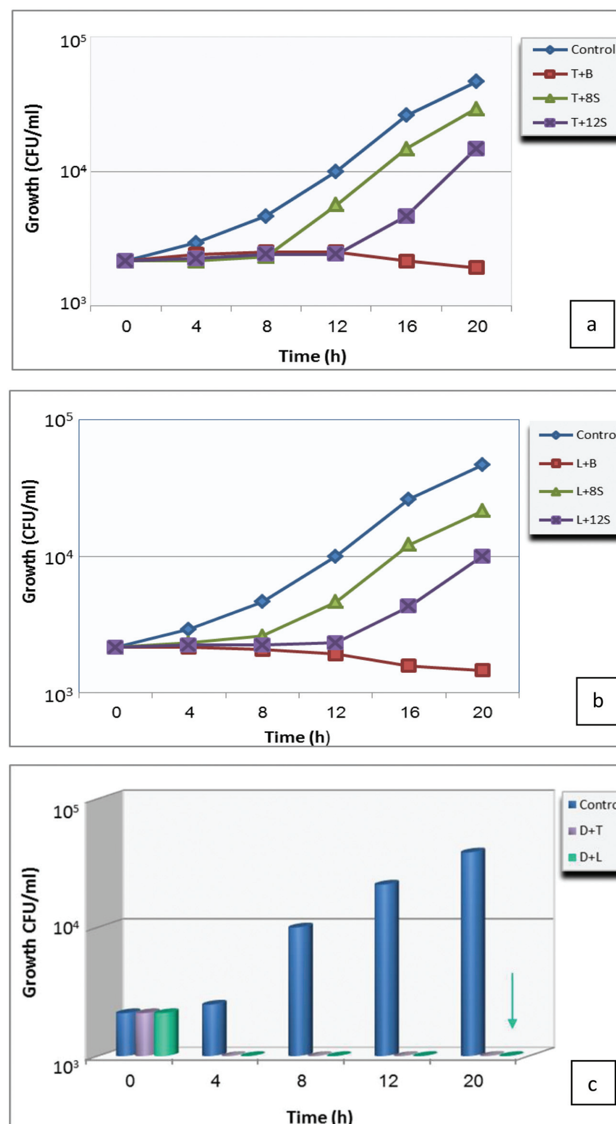
Both of iron-free pure LF and TF inhibited the growth of *S. pyogenes* in BHI broth medium; however, there

Figure 4



Bactericidal activity of human milk against growth of *Streptococcus pyogenes*. Symbols: control (DBHI, bacteria), SM+B (iron-saturated human milk, DBHI, and bacteria), FM+B (iron-free human milk, DBHI, and bacteria), and NM+B (natural human milk, DBHI, and bacteria). All experiments were carried out in triplicate and mean value was illustrated (*P*=0.319). The SDs were in the range of 2.6–3.3%. BHI, brain heart infusion.

Figure 5



Growth curve of *Streptococcus pyogenes* in iron-complete BHI broth (blue line) or in iron-depleted BHI broth with (a) Apo TF (red)+ iron-saturated TF after 8 h (green) and 12 h (violet); (b) Apo-LF (red)+iron-saturated LF after 8 h (green) and 12 h (violet), and (c) growth chart of *Streptococcus pyogenes* grown on deionized water (con) before transferred to completed BHI broth; deionized water+Apo lactoferrin (green) or Apo transferrin (violet). The SDs were in the range of 2.8–3.9%. BHI, brain heart infusion; LF, lactoferrin; TF, transferrin.

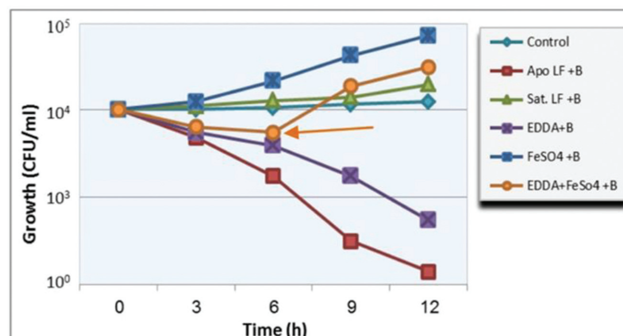
was no loss in viability. The addition of iron saturated LF or TF (Fig. 5a and b) after 8 and 12 h of inhibition led to further bacterial growth, revealing the inhibition of *S. pyogenes* was reversible in nature. These results indicated that both of Apo-LF and Apo TF showed only a bacteriostatic effect on *S. pyogenes*, which was owing to the binding of LF to the free iron causing iron deficiency that inhibited the growth [33]. Therefore, iron withholding by the host is a mechanism to prevent *S. pyogenes* infection [2,6].

In contrast to the aforementioned reversibility results, treatment of cells with Apo-LF or Apo TF in the absence of media resulted in an irreversible growth inhibition, where the cells completely lost their viability, as shown in Fig. 5c. These results indicated that both Apo-LF and Apo TF have a bactericidal effect against *S. pyogenes*. However, this protein displays antimicrobial properties against bacteria by limiting the availability of environmental iron, and it has a central role in host immunity [34]. However, in this case, the killing effect may not be related to iron deficiency [35]. Therefore, the antimicrobial activity here was not attributed to iron chelation but to other mechanisms such as the direct interaction with the pathogen where the positive charge of LF can interact with negative ones on the microbe and cause cell lysis [36].

In vivo study of the effect of lactoferrin on the survival of Streptococcus pyogenes

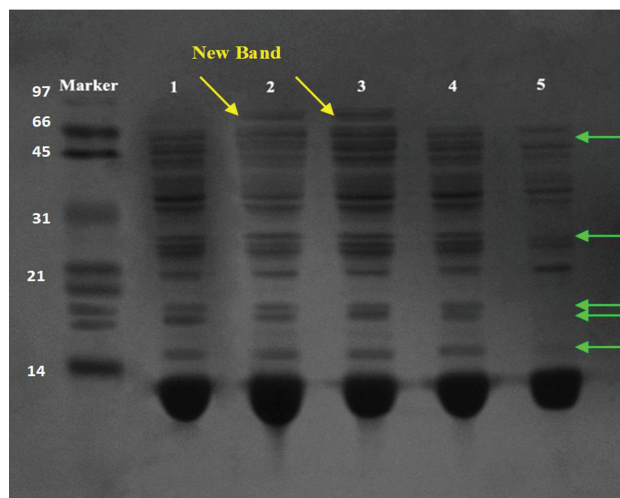
The *in vivo* study indicated that there was a considerable growth obtained when the mice were injected with $FeSO_4.7H_2O$. However, in the absence of iron, the bacterial cells maintained their count number. On the contrary, in the presence of iron-saturated LF, some minor increases were noticed, but the majority of cells were killed in the presence of either EDDA or iron-free pure Apo-LF. Injection of an excess amount of $FeSO_4.7H_2O$ in the EDDA preinjected mouse 6 h after bacterial injection led to a sharp increase in bacterial cells (arrow), as shown in Fig. 6. This may be owing to that when LF was in its iron-free state (Apo-LF), it was able to kill the pathogenic bacteria through its bactericidal effect. However, in case of iron-saturate LF, *S. pyogenes* may became resistant to LF because it is commonly

Figure 6



Effect of LF on survival and growth of *Streptococcus pyogenes* in mice. Symbols: control (saline, bacteria), saturated LF+B (iron-saturated LF, bacteria), Apo-LF+B (iron-free LF, bacteria), EDDA+B (EDDA, bacteria), $FeSo_4$+B ($FeSO_4$, bacteria), and EDDA+$FeSO_4$+B (after 6 h of bacteria inoculated add 1.25 mg EDDA+60 μg $FeSO_4$). All experiments were carried out in triplicate, and mean values were illustrated (P=0.008). The SDs were in the range of 2.4–3.5%. EDDA, ethylenediamine-di-o-hydroxyphenyl acetic acid; LF, lactoferrin.

Figure 7



SDS-PAGE lanes of surface proteins of *Streptococcus pyogenes*: (a) BHI broth media, (b) iron-depleted BHI media, (c) in the presence of EDDA, (d) in the presence of iron-saturated LF, and (e) in the presence of iron-free LF, and a low-molecular-weight marker on the left. BHI, brain heart infusion; EDDA, ethylenediamine-di-o-hydroxyphenyl acetic acid; LF, lactoferrin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

a part of the normal oral flora and it may also synthesize iron chelators that can compete with LF for iron [36]. These findings are consistent with the other studies which showed enhancement of *Yersinia pseudotuberculosis* viable count in the presence of iron-saturated LF, whereas the growth was reduced by Apo-LF and EDDA [16]. From the other side, the iron-free environment provided by LF in the cells of the host can function to kill pathogens and restore host tissue from infection as mentioned by Haversen *et al.* [37] who noted that human LF protected mice against urinary tract infections.

Isolation of surface proteins *of Streptococcus pyogenes*

Figure 7 shows the comparison of surface proteins of cells grown on BHI broth medium (control, Lane 1), iron-depleted BHI broth (Lane 2), EDDA (Lane 3), iron-saturated pure LF (Lane 4), and iron-free pure LF (Lane 5) added to the normal medium. The bands in the presence of iron-saturated LF (Lane 4) had similar patterns as the control (Lane 1), whereas Lane 2 and Lane 3 show a new protein band appeared upon growth under iron deficiency (yellow arrows). Lane 5 shows disappearance or decreased protein bands upon growth in the presence of iron-free LF (green arrows). This indicates that upon limited iron stores, cells are likely to express virulence factors that are required to adapt and survive under these conditions [27], as in case of Lane 2 and Lane 3, where a high-molecular-weight protein was induced by iron starvation and in the presence of iron-chelator EDDA. However, in case of Lane 5, iron starvation, as expected, affected the physiology of the microbe by regulating some surface proteins that are essential for bacterial adaptation to iron stress [38]. Furthermore, upon infection and bacterial virulence, the same attitude happened by *S. pyogenes*, which can express interaction proteins as virulence factors to evade the immune system, for adherence, and to acquire different metabolites, and many of these proteins are still poorly characterized [39]. Furthermore, Madeline *et al.* [40] confirmed that metal-limiting environment was considered as a critical factor for virulence. Therefore, to develop antibiotics or vaccines to treat the microbial infection, one must first know the strategy of bacteria to invade human body and induce infection.

Conclusion

This study confirmed several previous studies about *S. pyogenes* infection and provides additional evidence about the iron-related pathogenicity of this bacterium which became more clear when new proteins were emerged under iron-limitation environment, which could reflect the adaptation of the bacterium and consequently appearance of virulence. In addition, this may help in the future development of new therapies depending on studying the less tested surface-bound proteins that are responsible for bacterial virulence and their interaction with their host receptors in different host sites taking into consideration our previous results.

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Conflicts of interest

There is no conflict of interest.

References

1 Brouwer S, Timothy CB, Tania R, Manfred R, Mark JW. *Streptococcus pyogenes* adhesion and colonization. FEBS Lett 2016; 590:3739–3757.

2 Yamaguchi M, Terao Y, Kawabata S. Pleiotropic virulence factor – *Streptococcus pyogenes* fibronectin-binding proteins. Cell Microbiol 2013; 15:503–511.

3 Pankov R, Yamada KM. Fibronectin at a glance. J Cell Sci 2002; 115:3861–3863.

4 Grousd JA, Rich HE, Alcorn JF. Host-pathogen interactions in gram-positive bacterial pneumonia. Clin Microbiol Rev 2019; 32:1–21.

5 Crichton R. Iron metabolism: from molecular mechanisms to clinical consequences. John Wiley and Sons Ltd; 2009.

6 Akhter F, Edroyal W, Jorge E, Yoann L, Kevin SM, Shrikant P, Zehava E. Hemoglobin stimulates vigorous growth of *Streptococcus pneumoniae* and shapes the pathogen's global transcriptome. Sci Rep 2020; 10:15202.

7 Montanez GE, Neely MN, Eichenbaum Z. The streptococcal iron uptake (Siu) transporter is required for iron uptake and virulence in a zebra-fish infection model. Microbiology 2005; 1513749–1513757

8 Coombs MRP, Kronforst K, Levy O. Neonatal host defense against Staphylococcal infections. Clin Dev Immunol 2013; 2013:826303.

9 Weinberg ED. Iron loading in humans: a risk factor for enhanced morbidity and mortality. J Nutr Environ Med 2007; 16:43–51.

10 Memon BA, Abdul Latif S. Inhibition of growth of *Streptococuss pyogenes* and Escherichia coli by EDDA. J Chem Soc Pak 2006; 28:252–255.

11 Bates CS, Montañez GE, Woods CR, Vincent RM, Eichenbaum Z. Identification and characterization of a *Streptococcus pyogenes* operon involved in binding of hemoproteins and acquisition of iron. Infect Immun 2003; 711042–711055

12 Lemos ML, Balado M. Iron uptake mechanisms as key virulence factors in bacterial fish pathogens. J Appl Microbiol 2020; 129:104–115.

13 Nairz M, Haschka D, Demetz E, Weiss G. Iron at the interface of immunity and infection. J Front Pharmacol 2014; 5:152.

14 Weinberg ED. Iron availability and infection. Biochim Biophys Acta 2008; 1970:600–605.

15 Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA. Manual of clinical microbiology. 9th.ed. Washington, DC: American Society for Microbiology 2007.

16 Al-Salamah AA, Al-Obaidi AS. In vivo and in vitro effects of lactoferrin on Yersinia pseudotuberculosis. Microbiologica 1995; 18:267–274.

17 Rogers HJ. Iron binding catechols and virulence in *E.coli*. Infect Immun 1973; 7:445–456.

18 Hajishengallis G, Russell MW. Innate humoral defense factors. Mucosal Immunology 2015; 2015:251–270.

19 Homan J. Principles of laboratory animal care. NIH Publ 1985; 14:85–23.

20 Gill RE, Heffron F, Falkow S. Identification of the protein encoded by the transposable element Tn3 which is required for its transposition. Nature 1979; 282:797–801.

21 Al- Salamah AA, Charnetzky WT. A permeability mutant of *Y. pestis* with increased susceptibility to phagocytosis which retains potential for intraphagocytic growth and virulence. ACTA Microboiol Hung 1986; 33:203–213.

22 Patterson MJ. Streptococcus. In: Baron's medical microbiology. (4th ed). Galveston: University of Texas Medical Branch at Galveston 1996.

23 Nachamkin I, Lynch JR, Dalton HP. Evaluation of a rapid system for species identification of alpha-hemolytic streptococci. J Clin Microbiol 1982; 16:521–524.

24 Poutrel B, Ryniewicz HZ. Evaluation of the API 20 Strep system for species identification of streptococci isolated from bovine mastitis. J Clin Microbiol 1984; 19:213–214.

25 Al-Salamah S. Effect of iron binding proteins on growth and virulence of *Streptococcus agalactiae*. A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Department of Botany & Microbiology. King Saud University. Riyadh Am J Clin Nutr 2009; 77:1537S–1543SS.

26 Memon BA. Effect of EDDA on the growth on *Streptococcus milleri* and Staphylococcus aureus. J Chem Soc Pak 2008; 30:294–298.

27 Milne TS, Michell SL, Diaper H, Wikstro P, Svensson K, Oyston PC, Titball RW. A 55kDa hypothetical membrane protein is an iron regulated virulence factor of *Francisella tularensis* subsp. Novicida U112. Med Microbiol 2007; 56:1268–1276.

28 Espinas NA, Kobayashi K, Takahashi S, Mochizuki N, Masuda T. Evaluation of unbound free heme in plant cells by differential acetone extraction. Plant Cell Physiol 2012; 53:1344–1354.

29 Austin B, Austin DA. Bacterial fish pathogens. Disease of farmed and wild fish. Switzerland: Springer 2016.

30 Lönnerdal B. Nutritional and physiologic significance of human milk proteins. Am J Clin Nutr 2003; 77:1537S–1543S.

31 Kim DH. Bacterial sidrophores promote animal host iron acquisition and growth. Cell 2018; 175:311–312.

32 Li H, Li N, Xu Q, Xiao C, Wang H, Guo Z, Zhang J, Sun X, He Q. Lipoprotein FtsB in *Streptococcus pyogenes* binds ferrichrome in two steps with residues Tyr137 and Trp204 as critical ligands. PLoS ONE 2013; 8:1–8.

33 Gomez-Estaca J, Lopez de Lacey A, Lopez-Caballero ME, Gomez-Guillen MC, Montero P. Biodegradable gelatin-chitosan films incorporated with essential oils as antimicrobial agents for fish preservation. Food Microbiol 2010; 27:889–896.

34 Rosa L, Cutone A, Lepanto MS, Rosalba Paesano R, Valenti P. Lactoferrin: a natural glycoprotein involved in iron and inflammatory homeostasis. Int J Mol Sci 2017; 18:1–26.

35 Bullen JJ, Rogers HJ, Spalding PB, Ward CG. Natural resistance, iron and infection: a challenge for clinical medicine. J Med Microbiol 2006; 55:251–258.

36 García-Montoya IA, Cendón TS, Arévalo-Gallegos SA, Rascón-Cruz Q. Lactoferrin a multiple bioactive protein: an overview. Biochim Biophys Acta 2012; 1820:226–236.

37 Haversen LA, Baltzer L, Dolphin G, Hanson LA, Mattsby-Baltzer I. Anti-inflammatory activities of human lactoferrin in acute dextran sulphate-induced colitis in mice. Scand J Immunol 2003; 57:2–10.

38 Moumène A, Gonzalez-Rizzo S, Lefrançois T, Vachiéry N, Meyer DF. Iron starvation conditions upregulate ehrlichia ruminantium type IV secretion system, tr1transcription factor and map1genes family through the master regulatory protein ErxR. Front Cell Infect Microbiol 2018; 7:1–11.

39 Happonen L, Simon H, Gabriel SB, Christofer K, Therese de N, Hamed K, *et al.* A quantitative *Streptococcus pyogenes*-human protein-protein interaction map reveals localization of opsonizing antibodies. Nat Commun 2019; 10:2727.

40 Madeline SA, Kelly SD, Lindsey RB. Metal homeostasis in pathogenic Streptococci. Microorganisms 2022; 10:1501.