Porcine Hepcidin Exerts an Iron-Independent Bacteriostatic Activity Against Pathogenic Bacteria

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Abstract Hepcidin was first identified as an antimicrobial peptide and later demonstrated that hepcidin is the long sought hormone to regulate iron homeostasis in mammals. Though its iron regulatory function has been extensively investigated, the studies on its antimicrobial properties are limited. The aim of current study was to evaluate the antibacterial activity of synthetic porcine hepcidin (pHepc) in vitro against pathogen bacteria via radial diffusion, colony forming count, transmission electron microscopy and DNA binding assays. Our results showed that pHepc exerted little bactericidal activity, but possessed bacteriostatic activity by reducing the viable *E* [illegible] K88, *E.* [illegible] ATCC 25922, [illegible] ATCC 25923 and [illegible] CMCC 50013. pHepc-treated *E.* [illegible] K88 exhibited longer cells and cytoplasm unevenly distribution, while pHepc-treated [illegible] led to cytoplasm leakage and partly lysis of bacterial cells. Gel retardation assay showed the existence of the binding affinity of pHepc for DNA. In addition, pHepc retained the bacteriostatic activity in a wide range of pH value from 4.0 to 8.0 or in the presence of iron, respectively. Considering the high expression in response to infection and the bacteriostatic activity, pHepc may be an important defense molecule for pig health.

Keywords Porcine hepcidin · Bacteriostatic activity · Antimicrobial peptide · Iron

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Introduction

Hepcidin was initially purified from human serum and urine (Krause et al. 2000; Park et al. 2001). Now two biological functions have been determined: an antimicrobial activity against several bacteria and fungi, and a central role in the regulation of iron homeostasis through its binding to the iron exporter ferroportin (Ganz 2008; Nemeth et al. 2004). It was subsequently identified in many mammalian species and the structure of hepcidin, a 20–25 amino cysteine-rich peptide, is highly conserved, suggesting a key role in major biologic functions (Ganz 2003; Pigeon et al. 2001). Porcine hepcidin (pHepc) gene is 411 bp, encodes holoproteins of 82 amino acid residues and generates putative signal peptides of 25 residues, which contains eight cysteines, linked to form four disulfide bonds (Sang et al. 2006). Although some data indicate that pHepc are differentially regulated by in vivo Salmonella infection of pigs and iron chelation (Sang and Blecha 2009), direct involvement of pHepc in antimicrobial responses remains unknown.

As a defense antimicrobial peptide, hepcidins from mammals, amphibians, and fish have been shown to exhibit antimicrobial effects against various Gram-negative and Gram-positive bacteria (Alvarez et al. 2014; Krause et al. 2000). As a result, hepcidin is considered an important component of the innate immune defense system. However, few studies have described the antimicrobial activity of pHepc. Like human hepcidin, pHepc possesses eight cysteines in four disulfide bonds, which is reported to be important for their antimicrobial activity (Campopiano et al. 2004; Hocquellet et al. 2012). Therefore, the oxidized state of peptides with several cysteines, like hepcidin, is very important for its optimal conformation and antimicrobial activity (Alvarez et al. 2014). Most antimicrobial peptides exert their antimicrobial activity mainly by

disrupting the cell membranes of cellular pathogens (Yeaman and Yount 2003). This rather unspecific mode of action is suggested to be responsible for the broad-spectrum activity of many peptide antibiotics.

In the current study, we measured the antibacterial activity of pHepc against pathogen bacteria that are linked to diarrhea, enterocolitis and related intestinal diseases in pigs. The effect of pHepc on morphology of *E* K88 and were also studied using transmission electron microscopy (TEM). In addition, the influence of pH, serum and iron strength on pHepc antibacterial activity were described.

Materials and Methods

Peptide Synthesis

A 25 amino acid peptide corresponding to pHepc (DTHFPICIFCCGCCRKAICGMCCKT) was synthesized by standard solid-phase procedures with 9-fluorenylmethoxycarbonyl (Fmoc) methods using an Apex 396 peptide synthesizer (Aapptec, Louisville, KY, USA) by GL Biochem (Shanghai) Ltd (Sang et al. 2006). After assembly and cleavage from the resin using trifluoroacetic acid (TFA), the peptides were oxidized in phosphate buffer containing guanidine, EDTA, cysteine, and cystine to yield a complex mixture of disulfide isomers. The major isomer for 25 analogs synthesized were isolated by RP-HPLC and analyzed by NMR spectroscopy to confirm that they possessed the native hepcidin fold (Clark et al. 2011). Analysis of the synthetic peptides by Agilent 1200 Series HPLC (Agilent technologies, CA, USA) and Thermo Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) revealed a purity ≥95 %. Peptides were diluted in milli-Q water to obtain a stock solution of 10 mg/mL.

Antibacterial Activity

Antibacterial activity of pHepc was tested against *E* K88 (*E.* K88), *E* ATCC 25922 (*E.* ATCC 25922), ATCC 25923 ( ) and CMCC 50013 ( ). All bacteria were obtained from China General Microbiological Culture Collection Center (Beijing, China) and grown in Mueller Hinton broth (MHB, Difco Laboratories, USA) under aerobic conditions.

Bactericidal activities of pHepc against *E.* K88, and were evaluated by radial diffusion assays. Briefly, bacteria (4×10^6 CFU) were inoculated into warm 1 % (wt/vol) low electroendosmosis (EEO) agarose (Sigma) and poured into a polystyrene culture dish. A well (3 mm diameter) was punched into the solidified agarose layer. Either 5 μg of neomycin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) or 25 μg of pHepc (50 μL) was then added into the wells and allowed to diffuse into the agarose for 3 h at 37 °C. The plate was then overlaid with 6 % Trypton Soya broth (TSB) with 1 % low EEO agarose. After overnight incubation at 37 °C, clear zones of bacterial killing surrounding each well were measured. Colony counting assays were performed with *E.* K88, *E.* ATCC 25922, and (Kacprzyk et al. 2007). Bacterial (2×10^6 CFU/mL) were mixed with isovolumetric pHepc solution (0–128 μg/mL) in round bottom polypropylene 96-well plates and incubated for 3 h at 37 °C. After the incubation period, tenfold serially diluted bacterial suspension (10- to 1000-fold in PBS) were transferred onto Mueller Hinton Agar (MHA, Difco Laboratories, USA) plates after which colonies were counted after 24 h incubation at 37 °C.

Transmission Electron Microscopy (TEM)

Mid-logarithmic phase *E.* K88 and cells were treated with pHepc (final concentration is 32 and 16 μg/mL, respectively) for 1 h at 37 °C. After treatment, bacterial pellets were prefixed with 2.5 % glutaraldehyde in phosphate buffer (pH 7.2) overnight at 4 °C and postfixed with 1 % osmium tetroxide for 1 h. They were dehydrated in a graded series ethanol (50, 70, 80, 90, 95 and 100 %) and transferred to 100 % acetone for 20 min. The cells were immersed in acetone/durcupan resin (1:1 and 1:3) for 1–3 h, immersed in resin embedding agent overnight and embedded in durcupan resin at 60 °C. Ultra-thin sections (50 nm thick), stained with uranyl acetate and alkaline lead citrate, were observed using a Model JEM-1230 TEM (JEOL, Tokyo, Japan).

DNA Binding by Gel Electrophoresis

Plasmid DNA was purified from *E.* K88 using a plasmid extraction kit (Biospin Plasmid DNA Extraction Kit, Sangon, Shanghai). DNA was mixed with increasing amounts of pHepc in 10 μL binding buffer (5 % glycerol, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl and 50 μg/mL bovine serum albumin) (Liu et al. 2013), after which the mixture was incubated for 1 h at room temperature, and finally subjected to 1 % agarose gel electrophoresis in TBE buffer. The peptide and DNA mass ratio varied from 1:1 to 16:1. Gel retardation was visualized under UV illumination using a Bio-Rad Gel Documentation system (UK).

Effect of pH and Serum

Antibacterial activities of pHepc at various pH against *E.* K88 and were evaluated by a liquid microdilution

assay in 10 mM sodium-phosphate buffer (Maisetta et al. 2006). Aliquots of bacteria (2×10^6 CFU/mL) were mixed with indicated peptide in isovolumetric phosphate buffer solutions of various pH (pH 2.0, 4.0, 6.0, 7.0, 8.0 and 10.0). The final concentration of pHepc was 16 μg/mL for *E. [illegible]* K88 and 8 μg/mL for *[illegible]*. The viable colonies were counted after 2 h of incubation as outlined above. *[illegible]* and *[illegible]* were used to evaluate the antibacterial activities of pHepc in the presence of serum. To obtain porcine serum, blood was collected from a 5-day-old piglet (Duroc × Landrace × York, Xiaoshan Duroc Breeding Co., Ltd, Hangzhou) by venipuncture of an anterior vena cava in 5 mL vacutainer tubes (Corning Co., China) without anticoagulant following the procedure in Maartja et al. (2013). Fifty microliters of 2×10^6 CFU/mL bacteria were mixed with 50 μL milli-Q water, 50 μL pHepc, 50 μL porcine serum or 50 μL serum treated-pHepc, respectively. The final concentration of pHepc was 8 μg/mL for both bacteria. The negative control was 100 μL porcine serum solution, while the positive control was 100 μL bacteria suspension. The viable colonies were counted after 2 h of incubation. Experiments were carried out in triplicate and replicated three times.

Effect of Iron Ionic Strength

Antibacterial assays in the presence of iron were performed against *E. [illegible]* K88 and *[illegible]*. Firstly, the influence of iron on bacterial growth was assessed in the presence of varying concentrations of iron. Aliquots of approximately 2×10^6 CFU/mL bacterial suspensions were incubated with same volume of 1.25–625 μM (final concentration) ferric chloride ($FeCl_3$) for 2 h at 37 °C before detection. Secondly, bacteria were co-exposed to $FeCl_3$ (10, 20 or 78 μM) and pHepc (16 μg/mL for *E. [illegible]* and 8 μg/mL for *[illegible]*) for 90 min at 37 °C. The mixture was then incubated for 2 h at 37 °C followed by tenfold series dilutions before the suspension was plated onto MHA agar. All of the data was obtained by using colony counting assays as mentioned before.

Results

Antibacterial Activity of pHepc

To evaluate the antibacterial activities of pHepc in vitro, pathogen bacteria including *E. [illegible]* K88, *E. [illegible]* ATCC 25922, *[illegible]* and *[illegible]* were used. At tested concentration (500 μg/mL of pHepc), no clear zone was observed in pHepc-treated *E. [illegible]* K88, *[illegible]* and *[illegible]* (Fig. 1a). In contrast, neomycin (100 μg/mL) showed 14–16 mm of clear zone. It suggested that pHepc exerts no bactericidal activity against tested bacteria. Colony counting assay showed that pHepc was active against *E. [illegible]* K88, *E. [illegible]* ATCC 25922, *[illegible]* and *[illegible]* in a dose-dependent manner (Fig. 1b). Survivals of all tested bacteria were affected by pHepc. The most susceptible bacteria was *[illegible]*, where the surviving bacteria reduced 2 log in the number of CFU/mL at the highest concentration of pHepc. In contrast, pHepc caused a reduction by less than 1.5 log in the number of CFU/mL in other tested bacteria. However, pHepc only decreased the number of viable bacteria, but couldn't inhibit their growth completely.

Effect of pH and Serum on the Activity of pHepc

pHepc contains a histidine residue, which may exhibit a pH-dependent antimicrobial activity (Kacprzyk et al. 2007). We sought to evaluate the effect of pH on the antibacterial activity of pHepc against *E. [illegible]* K88 and *[illegible]*s. pHepc displayed its bacteriostatic activity against *E. [illegible]* K88 at pH 6.0–8.0 (Fig. 2a). In contrast, a slight wider range of pHs between 4.0 and 8.0 was available for pHepc to reduce the viable *[illegible]* (Fig. 2b). To determine whether physiologically relevant biological matrices influenced the potency of pHepc, its activity was assessed in the presence of piglet serum. Compared to the control group, serum increased the number of *[illegible]*s and *[illegible]* significantly (Fig. 2c, d). However, when pHepc was co-incubated with serum, it failed to inhibit the growth of both bacteria.

Effect of pHepc on the Ultrastructure of Bacteria

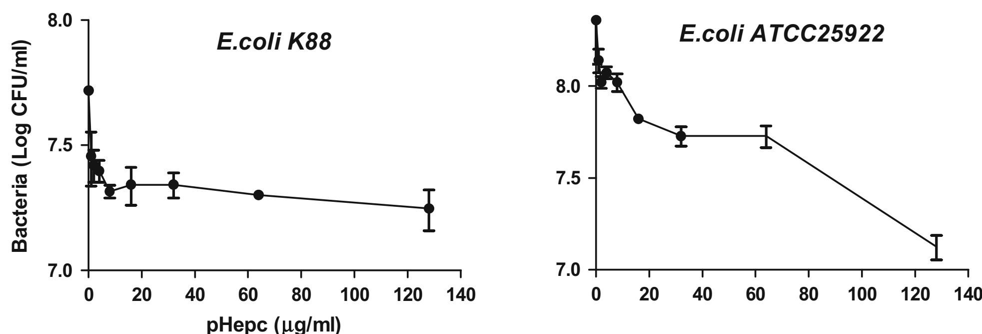
To elucidate the mechanism involved in pHepc mediated bacteriostatic activity, morphological changes in *E. [illegible]* K88 and *[illegible]* induced by pHepc were examined. After exposure to pHepc for 1 h, *E. [illegible]* K88 exhibited no big changes in cell shape, only some cells became longer and cell cytoplasm unevenly distributed inducing vacuole formation (Fig. 3); *[illegible]* appeared swollen, cell wall damage and vacuoles were seen in the core of the cell.

DNA Binding Assay

Gel retardation assay was used to evaluate the binding affinity of pHepc for plasmid DNA. When DNA and pHepc were mixed, the band shift occurred in ratio dependent (Fig. 4). The maximum band shift occurred at a mass ratio (pHepc:DNA) of 4, which indicated a formation of a peptide-DNA complex.

Effect of Iron on Bacteriostatic Activity of pHepc

In order to evaluate whether, due to its iron regulation function, the bacteriostatic effect of pHepc could be affected by the presence of iron, colony counting assay against *E. [illegible]* K88 or *[illegible]*

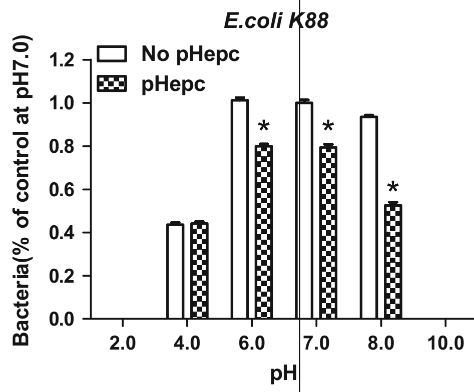


iron. In an initial experiment, where bacteria were incubated with $FeCl_3$, low concentrations of iron promoted the growth of bacteria, whereas high concentrations were the opposite (Fig. 5a, b). *E.* [illegible] K88 grew best in presence of 20 µM iron under current test conditions, while 78 µM iron was needed for best growth of [illegible]. In a second set of experiments, two bacterial species were co-incubated with pHepc (8 or 16 µg/mL) and growing concentrations of iron (10, 20 or 78 µM) (Fig. 5c, d). The results indicated that the presence of iron couldn't affect the bacteriostatic activity of pHepc.

Discussion

While the iron regulatory functions of pHepc have been extensively investigated (Lipinski et al. 2010; Starzynski et al. 2013), the antimicrobial properties of such peptide have not been explored in detail. Therefore, the aim of current study was to evaluate the antibacterial activity of synthetic pHepc in vitro against some pathogen bacteria and assess the effect of the presence of iron on its activity. Synthetic pHepc peptide inhibited growth of all tested bacteria in a dose-dependent manner across a wide range of pH conditions. Interestingly, pHepc exerts an iron-independent bacteriostatic activity.

Phylogenic analysis indicates that the mature peptide of pHepc is 84 % identical to human orthologs, and almost all residues defining hydrophobicity, net charge and intramolecular disulfide bridges (Sang et al. 2006). The functional consequences of the differences in the amino acid sequence of pHepc compared to human hepcidin (or other hepcidins) are hard to predict. In our study, pHepc


E.coli K88
No pHepc
pHepc
Bacteria(% of control at pH7.0)
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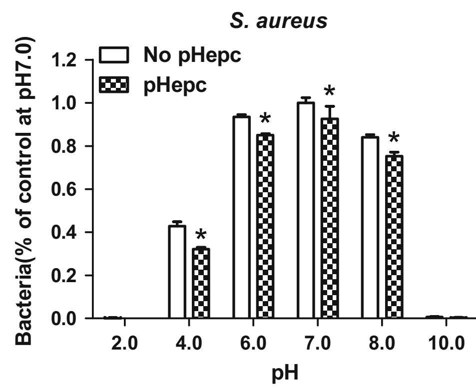
S. aureus
No pHepc
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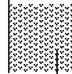
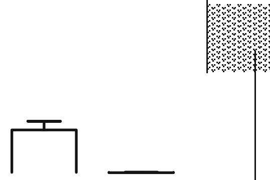



exerts no bactericidal activity against *E.* [illegible] K88, [illegible] and [illegible] due to the absence of clear zone in radial diffusion assay. However, colony count assay showed that pHepc decreased the survival number of *E.* [illegible] K88, *E.* [illegible] ATCC 25922, [illegible] and [illegible]. But it couldn't kill all of them. Some viable bacteria could still grow and replicated, which can explain why no clearance zone was observed after overnight incubation. It suggested that pHepc exhibited bacteriostatic activity, which was different from other hepcidins displaying bactericidal activity (Koliaraki et al. 2008; Maisetta et al. 2006).

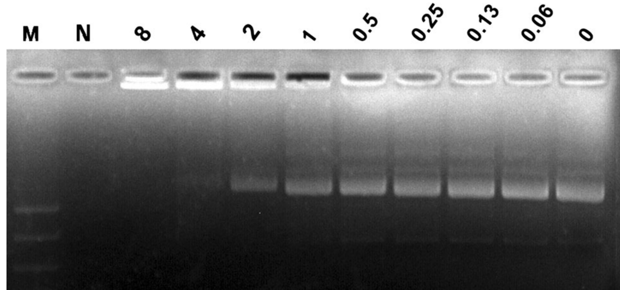
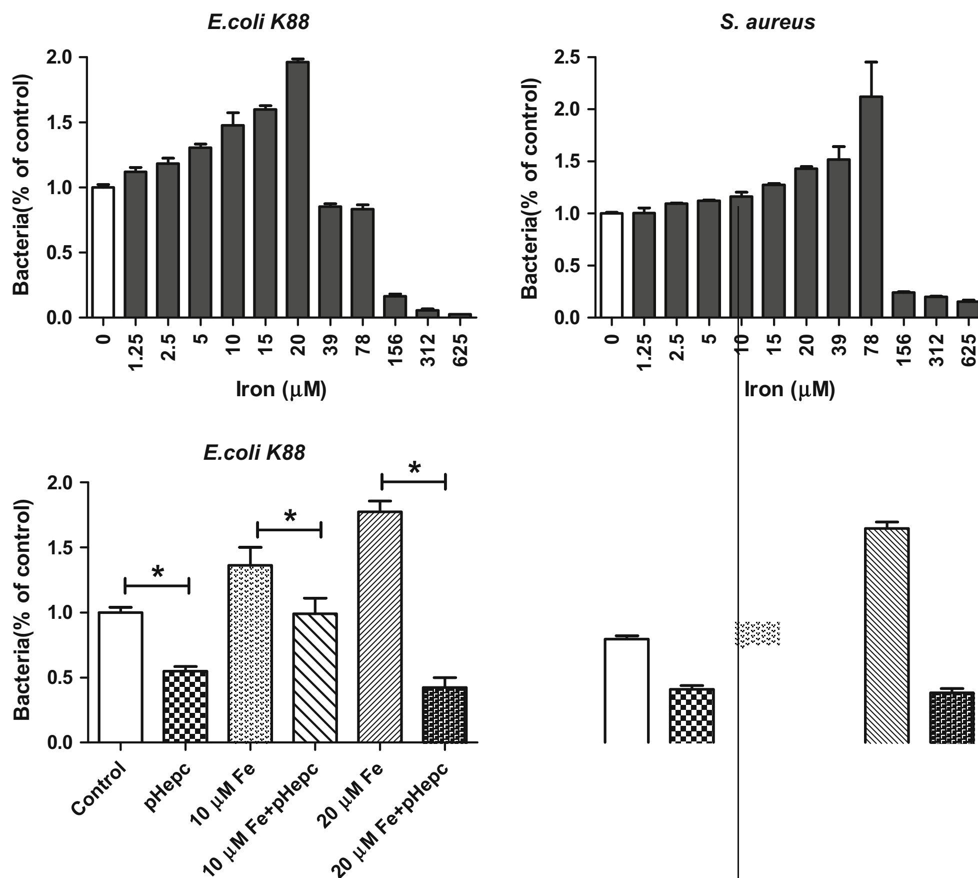


Fig. 4 Gel retardation assay showing the DNA-binding activity of pHepc. The mass ratio (peptide/DNA) was marked above each lane. *M* marker, *N* negative control

One of the major obstacles using cationic antimicrobial peptides as antimicrobial agents is the frequent finding that their antimicrobial activity can be strongly diminished under physiologically relevant conditions. To investigate the effect of pH and serum components on antibacterial activity, pHepc was tested against *E.* [illegible] K88, [illegible] and [illegible]. This result indicates that the solubility and self-association of pHepc are not much affected by pH (data not shown) and that the bacteriostatic activity of pHepc could be retained in the wide range of pH environments. In the sensitivity of pHepc to serum treatment, the peptide completely lost its bacteriostatic activity



against both bacteria after it was treated with porcine serum. This inactivation may be caused not only by binding of the peptides to serum albumin or other components in the serum (Kristensen et al. 2013), but also partial degradation of the peptides by serum proteases (Maisetta et al. 2008).

In order to elucidate the mechanism involved in pHepc mediated bactericidal activity, morphological changes of bacteria and DNA binding were examined. With the use of TEM, the effect of pHepc on the ultrastructure of *E.* [illegible] K88 and [illegible] was visualized. Vacuole formation, and retraction of the cytoplasmic membrane were the main effects to *E.* [illegible] K88, while to [illegible], cell swelling and cell lysis were observed. Since vacuole formation and retraction of the cytoplasm precedes cytoplasm leakage and lysis of cell, [illegible] cells might be more damaged than *E.* [illegible] K88 cells after treatment of pHepc. It seems that pHepc could widely damage cell membrane of [illegible] as little intact cell were seen when treated by pHepc. Furthermore, pHepc displayed strong binding affinity for plasmid DNA. It indicated that pHepc could act on intracellular targets. This binding ability of hepcidin may be dependent on the presence of the intramolecular disulfide bridges (Hocquellet et al. 2012).

Since hepcidin plays an important role in regulating iron homeostasis (Nemeth et al. 2004), it is unclear whether iron would affect the antibacterial activity of pHepc. Iron is an essential element in almost all organisms including bacteria, which is contained in many redox enzymes of the intermediary metabolism and in membrane-bound electron transport chains of respiratory systems (Braun and Hantke 2011). To identify the suitable concentraion of iron source for bacterial growth, various concentrations of $FeCl_3$ as iron sources were added to iron-deficient media. The results confirmed that iron is the essential nutrition for microbial growth at certain concentration and 20 or 78 μM iron was needed for best growth of *E.* [illegible] K88 and [illegible], respectively. To assess the hypothesis that iron affects the bacteriostatic activity of pHepc, colony counting assays were performed by exposing bacteria to both $FeCl_3$ and pHepc. The results demonstrated that pHepc remains its bacteriostatic activity against *E.* [illegible] *K88* and [illegible] at elevated iron concentrations.

It is believed that, following the induction of hepcidin expression, the primary antimicrobial activity of hepcidin in vivo may be that of limiting the availability of iron that is vital for invading microorganisms, thus contributing to host defenses (Ganz 2006). Here we provide another possibility. When piglet is infected by bacteria, among other antimicrobial peptides, pHepc can directly inhibit the growth of pathogen bacteria and give the time for wakening immune responses. Although pHepc doesn't work in the presence of serum, it could be useful in local infections.

In summary, synthetic pHepc possessed bacteriostatic activity against the tested bacteria and had good stability in a wide range of pH values except in serum. Though iron provides nutrition for microbe, the bacteriostatic activity of pHepc was not affected in presence of iron. The mechanism of its action illustrated that the survival of bacteria was decreased not only due to the vacuole formation, retractrion of the cytoplasm, cytoplasm leakage and lysis of cell, but also due to intracellular DNA binding. It indicated that pHepc could play an important role in innate immunity response of pigs against pathogenic bacteria in addition to its well-known role in iron regulation.

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Conflict of interest Dan Liu, Yutian Pu, Haitao Xiong, Yizhen Wang, and Huahua Du declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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