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Milk-derived antimicrobial peptides to protect against Neonatal Diarrheal Disease: An alternative to antibiotics

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Abstract

Neonatal Diarrheal Disease is responsible for significant economic losses to the livestock industries in Canada and around the world. Microbes responsible are diverse and include *Escherichia coli*, Salmonella, Rotavirus, Coronavirus and Cryptosporidia. While the use of antibiotics as a treatment for bacterial infections and as a prophylactic additive in feed has dramatically improved cattle production in recent decades, the increasing pressure to reduce or eliminate use of antibiotics in animals has caused the livestock industry to seek appropriate alternatives. Antimicrobial/Host Defense Peptides are natural compounds present on skin and in secretions in plants and animals that are microbicidal for bacteria, viruses, and parasites and they stimulate the immune system to combat infectious diseases. Our objective is to establish orally-obtained Host Defense Peptides (HDPs) as an alternative to antibiotics to protect against Neonatal Diarrheal Disease in calves. We devised a method to allow the cow udder to act as a factory to produce HDPs so that suckling calves will receive a continuous oral dose of HDPs over several weeks to protect them against neonatal diarrhea. We will use Adenovirus to deliver a gene coding for several HDPs in-frame into mammary epithelial cells. The epithelial cells will secrete the HDP protein into milk to be consumed by the suckling calves and trypsin in the calf gut will release the HDPs through cleavage. Thus, the novelty of this research lies not only in the proposed alternative to antibiotics to protect neonates against disease, but in the method by which we introduce the peptides to the suckling offspring.

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1. Introduction

Neonatal Diarrheal Disease is one of the leading causes of morbidity and mortality in dairy and beef calves and improved prevention and treatment methods would be of significant economic benefit to the cattle industry. The use of antibiotics as a treatment for bacterial infections and as a prophylactic additive in feed has dramatically improved cattle production in the last century, however, the rise of antibiotic-resistant bacteria and pressure to reduce or eliminate use of antibiotics has collectively driven livestock industries to seek appropriate alternatives.

Host Defense Peptides (HDPs; also known as antimicrobial peptides or innate defense regulators) are natural compounds present on skin and in secretions such as tears, saliva and milk in cattle and other animals. HDPs are naturally anti-bacterial, anti-viral and anti-parasitic, and they can also stimulate the immune system to combat infectious diseases [1-3]. Unlike antibiotics, HDPs are not disease-specific and can at once potentially protect against multiple bacterial, viral and parasitic challenges. In this report, we selected four HDPs (Indolicidin (Indol), Lactoferricin (LFn), Bovine Myeloid Antimicrobial Peptide-27 (BMAP-27), and Protegrin-1 (PG-1)) which have individually been shown to possess anti-microbial, anti-inflammatory or immunomodulatory activities [1, 4-7]. We evaluated their synergistic effects in induction of IL-8 expression, and indicator of innate immune activation, and their anti-microbial activity.

Supplementation of HDPs such as Lactoferrin into diet has been shown to protect calves against bacterial diseases suggesting that HDPs may be an effective replacement for antibiotics in feed [8]. However, adding synthetic peptides directly to feed or water as a prophylactic treatment and/or to treat disease is cost-prohibitive. To circumvent these costs, we devised a novel HDP delivery system: We intend to manipulate the cow's mammary glands to secrete significant quantities of select HDPs into milk using adenovirus gene delivery system. Through consumption of HDP-enriched milk, the calf will be supplemented with HDPs, which can then act directly as antimicrobial agents as well as influence the calf gastrointestinal immune system to protect against Neonatal Diarrheal Disease.

2. Materials and Methods

2.1. Cell lines and culture conditions

The bovine mammary epithelial cells (MAC-T cells) were obtained from the American Type Culture Collection (ATCC CRL-10274; Rockville, MD, USA) and cultured as indicated in [9].

2.2. Peptide synthesis

The following peptides were purchased from ChemPep (Wellington, Fl, USA): Indol (N-ILPWKWPWWPWRRG-C; GenBank: NM_174827), BMAP-27 (N-GRFKRFRKKFKKLFKKLSPVI-PLLHLG-C; GenBank: NM_174832), and PG-1(N-RGGRLCYCRRRFCVCVGR-C; GenBank: NM_001123149).

LFn (N-IEGRFKCRRWQWRMKKLGAPSITCVRRAF-C; GenBank: AAQ98614.1) was synthesized in-house on a PIONEER™ solid-phase peptide synthesizer (Applied Biosystems Life Sciences, Foster City, CA, USA) using Fmoc (9-fluorenylmethoxy carbonyl) chemistry. Peptide chains were synthesized from the carboxyl terminus to the amino terminus onto [5-(4-Fmoc-aminomethyl-3,5-dimethyloxyphenoxy) valeric acid]-polyethylene glycol-polystyrene (PAL-PEG-PS) resin (OVA (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). Fmoc-protecting groups at the amino terminus were deprotected with piperidine. The peptides were cleaved from the resin with concurrent deprotection of the

side chain-protecting groups by treating the resin-bound peptide with trifluoroacetic acid (TFA) (Sigma) (9.3 parts) in the presence of scavengers (anisole-ethyl-methyl sulfide-1,2-ethanedithiol [3:3:1]), for 4 h. The crude peptides were filtered from the resin, and the TFA was evaporated. Diethyl ether (Sigma) was added to the residues to precipitate the crude peptide. The peptides were isolated and purified by high-performance liquid chromatography (HPLC) on VYDAC® protein C_4 columns (1.0 by 25 cm) eluting with a linear gradient of 10% buffer A (H_2O–0.1% TFA)–90% buffer B (acetonitrile–H_2O [90/10]–0.01% TFA) for 40 min at a flow rate of 3 mL/min. Fractions of greater than 95% purity were analyzed on a 4800 Matrix Assisted Laser Desorption Ionisation (MALDI) Time of Flight (TOF)/TOF Analyzer Mass Spectrometer (Applied Biosystems Life Sciences, Carlsbad, CA, USA), at the National Research Council, Saskatoon, Saskatchewan, Canada. HDPs were diluted in sterile, pyrogen-free saline (Sigma).

2.3 Anti-microbial assay

Enterotoxigenic *E. coli* strain B44 (O9:K30;K99:H-) [10] was grown in minimal media (Vogel Bonner media; VB [11]) or Brain-Heart Infusion (BHI; Fisher) as indicated at 37°C in a shaking incubator until it reached log phase. A concentration of 10^5 cfu/ml was plated into a round-bottom, Nunc 96-well plate (Fisher Scientific) then incubated with the peptide cocktail (each peptide was included at 50 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 1.5 µg/ml or 0 µg/ml (media alone)) for 18 hrs at 37°C . The *E. coli* was then plated onto MacConkey agar plates (Fisher) the next day. Results were recorded as follows: (-) = no growth, (+) = low growth (very few colonies), (++) = medium growth, (+++) = heavy growth, (++++) = (very heavy growth).

2.4. Cellular viability assay

A Trypan blue exclusion assay was used to assess the cytotoxicity of the peptides on MAC-T cells. Cells were cultured in the presence of the peptides for 24 h before incubation with 0.025 % Trypan Blue (Invitrogen, Burlington, ON, CAN). The number of viable (clear) and dead (blue) cells were assessed by flow cytometry and presented in Table 1 as a percentage of viable (clear) cells in the presence of media or increasing concentration of HDPs.

2.5. IL-8 protein expression

IL-8 expression was quantified using DuoSet® ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.6. Gene design, cloning and replication-defective adenovirus construction and amplification.

Using their proprietary OptimumGene™ technology, we purchased a gene designed to code for the active regions of Indol, Lactoferrin, BMAP-27, PG-1 and FLAG (N-DYKDDDDK-C) from GenScript (Piscataway, NJ USA) (Supplementary Fig 1) and the gene (5'Signal-Indol/LFn/BMAP-27/PG-1-FLAG 3') was cloned into a CpG-free vector (pCpGfree-mcs Invivogen, San Diego, CA, USA) using BglII and NcoI restriction endonuclease sites (New England Biolabs Ltd., Pickering, ON, Canada). The gene was designed such that it had minimal CpG oligodeoxynucleotides and was optimized for high expression in sheep (i.e. sheep were chosen as our animal model of choice instead of calves due to lower costs).

2.7. Adenovirus-mediated cloning and transduction.

E1-substituted Ad5 Adenovirus cloning was performed by O.D.260, Inc. as detailed in [12]. Recombinant adenovirus vectors were amplified in 293 cells (ATCC; CRL-1573). Cells were cultured in DMEM (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (FCS) (Sigma), 4 mM glutamine, 1% penicillin-streptomycin (Life Technologies), and 4.5 mg glucose/L. CsCl-

purified virus was dialyzed against GTS buffer (2.5% glycerol, 25 mM NaCl, 20 mM Tris-HCl pH 8.0; (Sigma)) and filtered through a 0.22 μm Millex-GV membrane (Fisher Scientific, Mississauga, ON, Canada). Infectious virus titres were determined using TCID50 assay [13].

2.8. Dot-Blot Analysis.

The 293 cells were plated in 6 well Nunc plates (Nalgene) for 18 hr prior to transduction with 2 μl crude extract of Adenovirus coding for Indol/LFn/BMAP-27/PG-1-FLAG in 2 ml total volume. Seventy-two hours later, supernatants were collected and frozen at -80 ^{o}C. Supernatants were evaporated by Speed-Vac to approximately 200 μl volume and 10 % was added to methanol-treated, dried, PVDF membrane. Five μg of 293 cellular supernatants which had previously been transfected with bacterial plasmid expressing FLAG (a kind gift from McFie and Stone [14]) was also blotted onto the membrane and was allowed to dry at room temperature. The blot was blocked with 1 % skim milk in 0.05 %Tween 20 in Tris-buffered saline (TBST). TBST buffer for 1 hour. Primary rabbit anti-FLAG antibody (1 μg/ml; Genscript, Piscataway, NJ, USA) was incubated with the blot at room temperature with gentle shaking for 2 hr. After washing 3 times with 5 ml of 1% skim milk/TBST, the secondary Alkaline-phosphatase-conjugated goat-anti-rabbit antibody (KPL, Gaithersburg, MD, USA) was incubated for 1 hour at room temperature gentle shaking. Blots were washed 3 times with 1% skim milk in TBST for 5 minutes each followed by three washes in TBST. Blots were washed 4 times with ddH$_2$O then 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP) (Sigma) insoluble alkaline substrate solution was added as per manufacturer's instruction.

3. Results and discussion

Because our ultimate goal is adenovirus-mediated HDP expression into cow milk for consumption by the calf, we anticipate that there will be a significant dilution effect in the milk and the large surface area of the gut. Therefore, we sought to identify a cocktail of HDPs which are effective at low concentrations. We evaluated the microbicidal activity of Indol, LFn, BMAP-27, and PG-1 alone and in combination against an *E. coli K99* bovine field isolate known to cause Neonatal Diarrheal Disease in cattle [10]. Individually and in the majority of combinations, Indol, LFn, BMAP-27, and PG-1 were poorly microbicidal at 1.5 μg/ml (Table 1). However, when Indol/LFn/BMAP-27/PG-1-FLAG were introduced to the bacteria at 1.5 μg/ml each, the cocktail proved to be strongly anti-microbial. A trypan blue exclusion assay determined that even when Indol/LFn/BMAP-27/PG-1 were at 12. 5 μg/ml, they were not cytotoxic to MAC-T cells (Table 2). Next, we evaluated the effect that Indol/LFn/BMAP-27/PG-1 had on IL-8 protein expression, a chemokine known to induce both chemotaxis and activation of neutrophils [15, 16] and an indicator or innate immune activation. Although this experiment needs to be repeated to establish whether these results are statistically significant, Indol/LFn/BMAP-27/PG-1 (1.5 μg/ml) prompted a moderate induction of IL-8 expression in MAC-T cells. In the presence of LPS, Indol/LFn/BMAP-27/PG-1 showed a strongly synergistic effect with production of > 3,000 pg/ml IL-8 after 48 hours. Thus, we have chosen to clone Indol/LFn/BMAP-27/PG-1 into adenovirus for further analysis.

To circumvent the costs associated with HDP production, we devised a procedure where-in the mammary glands of the cow will act to produce the HDPs which will be supplied to the suckling calf through the milk. We designed a gene such that Indol's natural signal peptide sequence was in-frame to the active region of Indol which was also in-frame with the active sequences for LFn, BMAP-27, PG-1 and FLAG. Because there are no commercially available antibodies against Indol, LFn, BMAP-27 or PG-1, we included the FLAG epitope as part of the gene product. Detection of FLAG using anti-FLAG antibodies could be used to infer expression of Indol, LFn, BMAP-27 and PG-1. The gene was designed

so that each HDP were separated from each other by four codons coding for Arginine. When translated into a protein, the HDPs will be freed from each other through trypsin digestion. This gene was synthesized as indicated in Materials and Methods and cloned into a CpG-free expression plasmid. In turn, the gene was sub-cloned into a replication defective adenovirus. When 293 cells were transduced with the modified adenovirus, we detected FLAG expression through dot-blot analysis suggesting that the peptides were also being expressed.

In future experiments, we intend to evaluate whether adenovirus-derived HDPs, like their synthetic counterparts, are strongly anti-microbial and promote IL-8 expression from MAC-T cells when co-stimulated with LPS. Once satisfied that the adenovirus-derived peptides are functional, we will transduce udders directly through the teat canal and then monitor the milk for the HDP/FLAG protein using antibodies against FLAG. Once consumed by the calf, the HDP/FLAG protein will travel to the trypsin-rich gut and, through enzymatic digestion, Indol, LFn, BMAP-27 and PG-1:FLAG will be released. Calf's will be infected with *E. coli K99* to establish whether continuous exposure to adenovirus-derived HDPs protects them against Neonatal Diarrheal Disease.

4. Conclusions

In this study, Indol/LFn/BMAP-27/PG-1 were shown to be strongly antimicrobial against *E. coli K99* but not cytotoxic to bovine mammary epithelial cells. The peptide cocktail synergized with LPS to promote robust quantities of IL-8 protein from MAC-T cells. Together, these results suggest that Indol/LFn/BMAP-27/PG-1 are strongly immunomodulatory and anti-microbial. Our next steps will be to establish adenovirus-mediated expression of Indol/LFn/BMAP-27/PG-1 in cow milk, and to determine whether it protect calves against Neonatal Diarrheal Disease.

Table 1. Minimum Inhibitory Concentration (MIC) Assay

Peptide Cocktail Composition*	Growth**
Media,	++++
Indol	++++
BMAP-27	++++
LFn	++++
PG-1	++++
Indol, LFn	++++
Indol, BMAP-27	++++
Indol, PG-1	++++
LFn, BMAP-27	++++
LFn, PG-1	++++
BMAP-27, PG-1	++++
Indol, LFn, PG-1	++++
Indol, BMAP-27, PG-1	++++
Indol, LFn, BMAP-27, PG-1***	-

* The concentration of each peptide was 1.5 μg/ml.
** *E. coli* was grown in BHI media.
*** *E. coli* was also grown VB media with similar results

Table 2. Trypan Blue Viability Assay

Peptide Cocktail Composition	Peptide Conc. (μg/ml)	Percent Viability (%)*
Indol, LF, BMAP-27 and PG-1	0	96
	0.75	96
	1.50	100
	3.13	96
	6.25	93
	12.50	97

*Cytotoxicity was assessed using MAC-T cells.

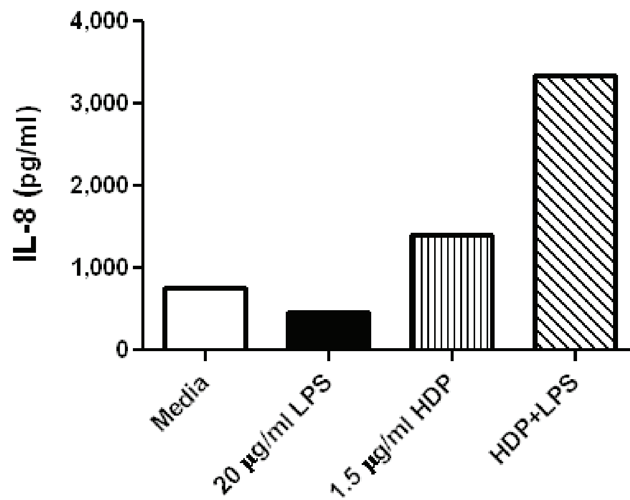

Fig 1. HDP cocktail-induced IL-8 protein expression. Bovine MAC-T epithelial cells were incubated for 72 hr with 20 µg/ml LPS, 1.5 µg/ml Indol/LFn/BMAP-27/PG-1 (HDP), LPS + 1.5 µg/ml Indol/LFn/BMAP-27/PG-1 or media alone. Il-8 protein production was measured in the supernatants using ELISA analysis. Although preliminary, these data indicate that HDP+LPS trigger robust production of IL-8 from mammary epithelial cells (n=1).

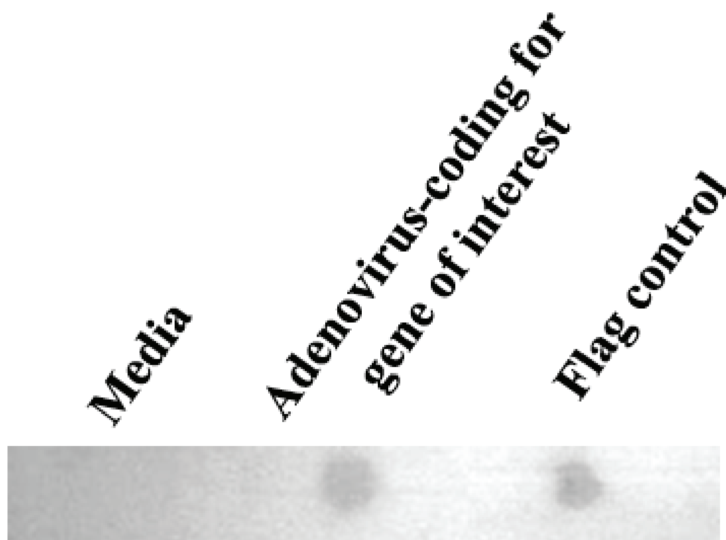

Fig 2. Dot-blot hybridization to detect FLAG epitope. Adenovirus coding for the HDP gene of interest was used to transduce 293 cells and supernatant was collected after 72 hours. For the positive control, 5 µg of total protein obtained from supernatants from 293 cell transfected with bacterial plasmid DNA coding for FLAG, was used. Supernatants were blotted onto PVDF membrane and hybridized with anti-FLAG antibody. Adenovirus transduced 293 cells expressed FLAG and therefore must also produce the HDP active regions.

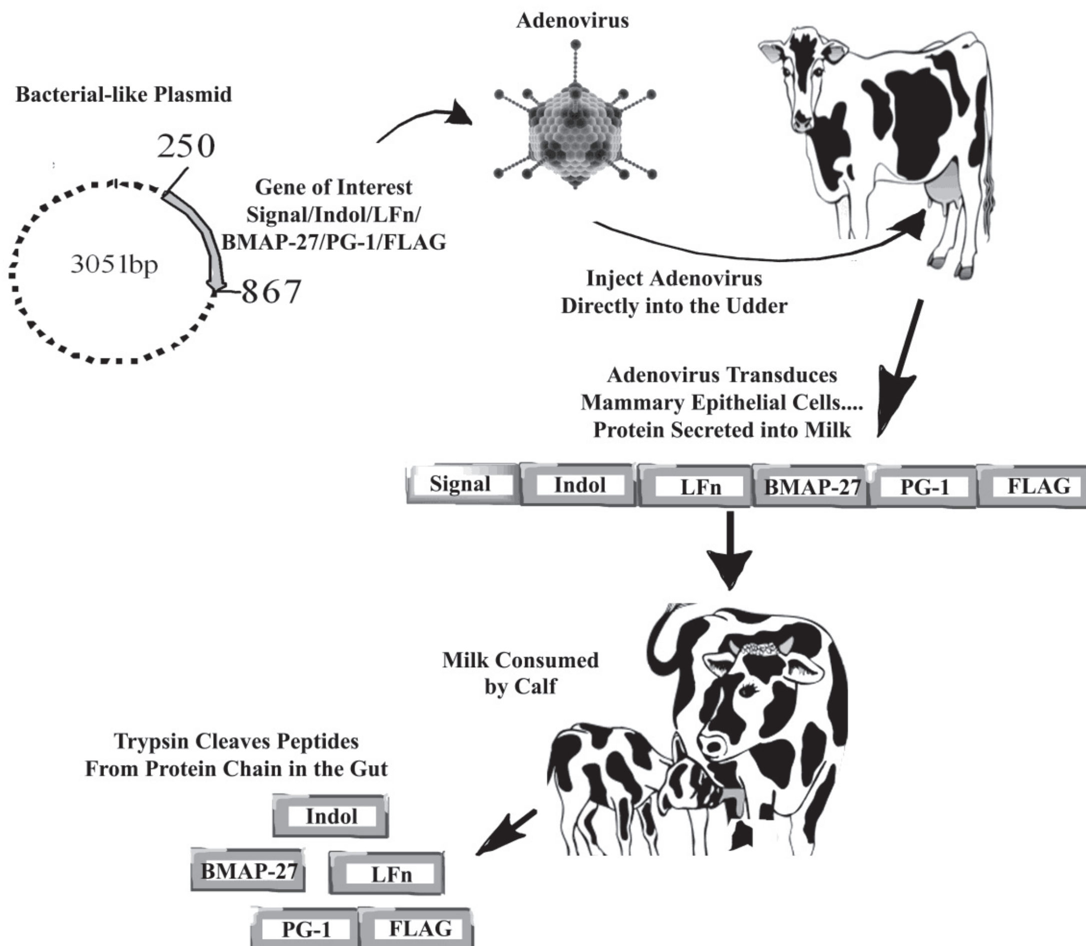

Fig 3: Schematic representation of the transfer of active HDPs to the calf gut via adenovirus-mediated gene expression in cow milk. The active portion of Indol/LFn/BMAP-27 and PG-1 were cloned in-frame to an bacterial-like plasmid which was in turn be introduced to an adenovirus vector. The gene includes a sequence corresponding to the Sequence Recognition Particle (Signal) to ensure secretion of the protein outside of the cell and a FLAG tag for detection and quantification purposes. The adenovirus will be injected into the mammary tissue through the teat canal with the intent that it will transduce the mammary epithelial cells which will, in turn, generate the HDP protein into the milk. Upon consumption by the calf, the HDP protein will travel to the gut where trypsin will cleave the HDP protein to release the active peptides. Images were obtained through public domain sites www.clker.com/clipart and www.imagefree.org.

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Appendix A: Supplementary Figure.

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  1  agatctatgc agacccagcg tgcttccctc tccctgggcc ggtggtccct gtggctcctg
             m   q  t  q   r  a  s   l  s  l   g  r  w   s  l  w   l  l

 61  ctcctcgggc tcgtggtgcc ttccgcctcc gcacaggctc tcagttaccg agaggccgtg
      l  l  g   l  v  v   p  s  a   s  a  q   a  l  s   y  r  e   a  v

121  ctgcgagctg tggaccagct gaacgagctc agctccgaag caaacctcta tagactgctc
      l  r  a   v  d  q   l  n  e   l  s  s   e  a  n   l  y  r   l  l

181  gagctggatc cccctccaaa ggacaacgag gacctgggca ccagaaagcc agtgtccttc
      e  l  d   p  p  p   k  d  n   e  d  l   g  t  r   k  p  v   s  f

241  accgtgaaag agacagtgtg ccctcgcaca atccagcagc cagccgagca gtgtgacttt
      t  v  k   e  t  v   c  p  r   t  i  q   q  p  a   e  q  c   d  f

301  aaggaaaagg gtagggtgaa gcagtgcgtg gggaccgtga cactggatcc cagtaacgac
      k  e  k   g  r  v   k  q  c   v  g  t   v  t  l   d  p  s   n  d

361  cagttcgatc tgaactgtaa cgagctccag tctgtgatcc tgccttggaa gtggccctgg
      q  f  d   l  n  c   n  e  l   q  s  v   i  l  p   w  k  w   p  w

421  tggccttgga ggagaggccg ccggcgacgt atcgaaggca ggttcaagtg taggagatgg
      w  p  w   r  r  g   r  r  r   r  i  e   g  r  f   k  c  r   r  w

481  cagtggcgaa tgaagaaact gggtgcacca agcatcacct gcgtgcgccg agcctttcga
      q  w  r   m  k  k   l  g  a   p  s  i   t  c  v   r  r  a   f  r

541  cgtaggagag ggcggttcaa gagatttcgc aagaaattca agaagctgtt taagaagctc
      r  r  r   g  r  f   k  r  f   r  k  k   f  k  k   l  f  k   k  l

601  tccccagtga tcccactgct ccacctcgga cgccggcgac gtaggggcgg aaggctgtgc
      s  p  v   i  p  l   l  h  l   g  r  r   r  r  r   g  g  r   l  c

661  tactgtagac ggcgtttctg cgtgtgcgtg ggacgggatt acaaagacga tgacgacaag
      y  c  r   r  r  f   c  v  c   v  g  r   d  y  k   d  d  d   d  k

721  tagtagccat gg
      *  *
```

Supplementary Figure 1. Nucleotide and predicted amino acid sequence of the synthetic gene construct designed by GenScript, Ltd. The nucleotide sequence is numbered on the left and the one-letter code for each amino acid is listed below the codon. The BglII and NcoI restriction endonuclease sites flanking the gene are larger font. The Signal recognition sequence for Indolicidin is in-frame at the 5'-end of the gene to ensure that the translated sequence is targeted for secretion. The amino acid sequences for Indol, BMAP-27, LFn and PG-1 active sites are in bold and underlined font. The sequence for the FLAG peptide is within a boxed region. The arginine rich trypsin peptidase cleavage sites separating the HDP sequences are in normal font. The stop codons are marked with an asterisk.