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The chicken host peptides, gallinacins 4, 7, and 9 have antimicrobial activity against *Salmonella* serovars

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Abstract

The gallinacin genes clustered on chromosome 3 of the chicken (*Gallus gallus domesticus*) genome encode a group of cationic antimicrobial peptides characteristic of the β-defensins. In this study, gallinacins 4, 7, and 9, all predicted to contain the conserved pattern of cysteines typical of β-defensins but differing in their charge and hydrophobicity, were characterised for their in vivo gene expression patterns and in vitro antimicrobial activities against *Salmonella* serovars. Reverse-transcription PCR analyses of chicken epithelial tissues indicated gallinacin (Gal) 7 expression to be ubiquitous while Gal 4 and Gal 9 expression appeared localized to specific epithelial tissues including the ovary, trachea, and lung, respectively. In addition Gal 7, but neither Gal 4 nor Gal 9, expression was identified in tissues taken from the non-domesticated bird species, *Parus caeruleus*, *Larus argentatus*, and *Columba palambus*. Analysis of Gal 7 expression in chickens in response to an oral challenge with either *Salmonella enterica* serovar Typhimurium SL1344 or *Salmonella enteriditis* indicated no significant increase in small intestinal Gal 7 mRNA expression although a significant increase ($p < 0.05$) was detected in the liver, suggesting that, in response to *Salmonella* infection Gal 7 expression is inducible in the liver. Neither Gal 4 nor Gal 9 expression was induced in the chicken small intestine in response to the oral *Salmonella* infection. The antimicrobial capabilities of Gals 4, 7, and 9 against *Salmonella* serovars including *S. typhimurium* SL*1344* and *S. enteriditis* were investigated in vitro using recombinant His-tagged peptides and a time-kill assay. The antimicrobial activity data indicated the potency of the recombinant gallinacins against the *Salmonella* serovars to be in the order Gal 9 $\geqslant$ 4 > 7, and provided evidence for the synergistic interaction of Gals 7 and 9 against *S. enteriditis*. These results support *in silico* data that Gals 4, 7, and 9 are part of the innate defences of the chicken and function in microbial killing activities.

Keywords: Avian; Gallinacins; *Salmonella*; Cationic antimicrobial peptide

Cationic antimicrobial peptides (CAMPs) are now widely accepted as an important component of the host innate immune system. It has also been suggested that the array of CAMPs in a host species may play a role in determining the pathogenicity of a microbe in that species [1,2]. As birds, both domesticated and wild, are universally recognised as a major reservoir of human enteropathogens including *Salmonella,* but are themselves often asymptomatic, an understanding of their CAMP profile may provide essential information on the mechanisms by which such organisms are able to colonise the avian gastrointestinal tract.

To date, over 800 CAMPs have been identified (http://www.bbcm.units.it/~tossi/amsdb.html). A key group, synthesized by vertebrates and invertebrates alike, is the defensin family [3,4]. This family of peptides is composed of small <10 kDa, cysteine rich cationic molecules with broad-spectrum activity against bacteria, fungi and enveloped viruses. Each defensin is typified by six conserved cysteines, and according to its pattern of di-sulphide bonding, can be sub-divided into one of three sub-families, namely α-, β-, and θ-defensins. β-Defensins are found in invertebrates as well as vertebrates and the six conserved

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cysteines fold to form three disulphide bonds in a C1–C5, C2–C4, and C3–C6 conformation. In general, β-defensins are synthesized by epithelial cells although they have also been identified in bovine neutrophils and avian leukocytes [5–7].

The β-defensins comprise a N-terminal signal sequence, indicative of their secretion, and a C-terminal mature antimicrobial peptide, the latter comprising discrete groups of cationic and hydrophobic amino acids. It is this structural conformation which appears key to their antimicrobial activity as the peptides are proposed to function by binding to the negatively charged surface components of bacterial membranes via electrostatic charge interactions, integrating into the membranes causing depolarization and microbial death [8,9]. The preferential targeting of bacterial membranes can probably be explained by the neutrality of eukaryote cell membranes. In addition to a killing role, human β-defensins have also been shown to promote other responses including chemotaxis and cytokine production, effects which probably accelerate bacterial clearance during periods of infection, and suggest CAMPs also function to link the host innate and adaptive systems [10].

Gals 1 and 2, were first purified from chicken heterophils [5,6] and shown to have activity against a number of common enteropathogens, including *Salmonella enteriditis* and *Campylobacter jejuni* [5]. Gal 3 expression was identified primarily in chicken epithelial tissues including the tongue and large intestine [11], while recently high expression of Gal 6 has been reported in the chicken oesophagus and crop compared to moderate expression in the gastrointestinal tract [12]. To date 13 chicken (*Gallus gallus domesticus*) genes clustered on chromosome 3 of the chicken genome CAMPs have been reported, more recently through in silico analyses [13,14]. While the application of gallinacins as potential molecular markers for poultry breeding has been reported [15], there is still a paucity of information regarding the antimicrobial activities and roles of these host defence peptides in the avian innate immune system. Thus focussing on the three gallinacins, 4, 7, and 9, which differ in their charge and hydrophobicity, we report on their in vivo expression patterns and their in vitro antimicrobial activities against *Salmonella* serovars.

Materials and methods

Molecular analyses

Three chicken EST clones (ChEST192f2, 203o22, and 924g16) encoding Gals 4, 7, and 9 (Accession Nos. AAS99315, AAS99318, and AAS99320), respectively, were obtained from MRC Geneservice, UK. The forward and reverse primers designed to amplify the coding regions of the cDNAs and the optimal annealing temperatures used in the amplification experiments are presented in Table 1.

Livestock

The chickens (Goldline) used in the experiments were supplied by the Comparative Biology Centre, Newcastle University and wild bird tissues were made available through natural control programmes. The in vivo challenge experiment was carried out under UK Animal Project license no. PPL60/2702. Two groups of 5-day-old female chicks were orally gavaged with 0.1 ml inoculum containing approximately 5×10^6 organisms of either *Salmonella enterica Typhimurium* SL*1344* or *Salmonella enteriditis*. Age-matched, non-infected controls birds were housed under similar environmental conditions and gavaged with 0.1 ml phosphate buffered saline (PBS). Chicks were sacrificed by cervical dislocation on the fourth day after infection. Tissue samples were collected, snap frozen in liquid nitrogen and stored at −80 °C until analysed.

Tissue expression

RT-PCR analyses. Total RNA was extracted from the avian tissues using Trizol (Biogenesis) according to the manufacturer's instructions. To reduce degradation RNAase inhibitor (Invitrogen) was added to each sample (1 U/μg RNA) before storage at −80 °C. All samples were pretreated, before reverse-transcription (RT), with DNAase (Promega) at a concentration of 1 U/μg RNA. RT-PCR analyses, using the appropriate optimal annealing temperatures described in Table 1, were performed as described previously [16]. The RT products were resolved by electrophoresis using either 1% or 1.5% TBE agarose gels with added ethidium bromide and photographed under UV illumination. In control samples reverse transcriptase was omitted to demonstrate that PCR amplification was not due to contamination with genomic DNA. Each RT product was verified by DNA sequencing.

Semi-quantitative RT-PCR. Co-amplification of Gal 7 and 18 S rRNA was carried out using the GeneAmp RNA PCR Core Kit (Applied Biosystems). The optimum conditions were established using the Gal 7 (Table 1) and 18 S rRNA-specific primers (Ambion), according to the manufacturer's instructions and as described previously [17]. Products

Table 1
Primer sequences used for reverse-transcription PCR analyses of avian tissues, and amplification and cloning of recombinant clones (Gal 4, 7, and 9 Rec) for hyper-expression studies

Gene	*T* (°C)	Primer sequence		Product size (Kbp)	
		Forward	Reverse	cDNA	Genomic
Gallinacin 4	55	CCGTGGCCATGAGGATC	GCGAATTCTCAGGCCC	0.2	4.4
Gallinacin 7	55	GTAGGTGGACAACATCT	CAACCATCTACAGCAA	0.3	0.9
Gallinacin 9	57.5	CAGCCATGCAGATCCTG	CGAATTCTCAGGAATAC	0.2	1.0
Gallinacin 4Rec	58	CGCGGATCCCAGCCCTA	CGCGAATTCTCAGGCCCAC		
Gallinacin 7Rec	60	CGCGGATCCTTTTCCCGTT	CGCGAATTCCTACAACCATC		
Gallinacin 9Rec	59	CGCGGATCCCGAGGATTA	CGCGAATTCTCAGGAATA		

In the latter the forward primers contain a *Bam*HI restriction site and the reverse primers an *Eco*R1 restriction site, which are underlined. *T* (°C) represents the optimum annealing temperature for PCR analyses.

were separated using 1.2 % TBE agarose gels with added ethidium bromide. Densitometry was performed using a GDS 5000 Gel documentation system (UV Products, Cambridge).

Peptide expression

DNA fragments encoding the putative Gal 4, 7 and 9 peptides were amplified by PCR using the primers (Gals 4, 7 and 9 Rec) listed in Table 1. Each fragment was cloned into a pRSET A expression vector (Invitrogen), transformed into Escherichia coli Origami B pLysS and induced using 1 mM IPTG (isopropylthio-β-galactoside)[16]. After an over-night incubation at 16 °C the cells were harvested by centrifugation, resuspended in 20 mM Tris, pH 8, and 1 mM Tris (2-carboxyethyl) phosphine (TCEP) (Sigma), and lysed by sonication. The soluble fractions were recovered, and the recombinant His-tagged peptides analysed by SDS–PAGE on 15% (w/v) polyacrylamide gels. The recombinant peptides were stored at −20 °C. Total protein concentrations were quantitated using a Bradford assay.

Anti-microbial assay

Time-kill anti-microbial assays, as described previously, were used to investigate the antimicrobial activity of the His tagged recombinant peptides against the following strains: *Salmonella enterica* serovar Typhimurium *phoP*, *S. enterica* Typhimurium SL1344 and *S. enteriditis* [16]. For each assay 10 μl of 200 mg/ml magainin (Sigma) was used as the antimicrobial positive control.

Haemolysis assay

Erythrocytes were extracted from chicken blood, washed three times in PBS, diluted to a concentration of 3% in PBS containing 10% serum, and 180 μl aliquots dispensed into 96-well plates. Increasing recombinant peptide concentrations in a final volume of 20 μl were added in triplicate to cells and incubated for 1 h at 37 °C. Following centrifugation at 1000*g* for 10 min, the supernatants were transferred to new 96-well plates and the absorbance measured at 560 nm. Controls for 0% and 100% haemolysis consisted of cells suspended in PBS only and in 0.2 % Triton X-100, respectively. The percentage haemolysis was calculated as described in [18].

Statistics

Data are expressed as means ± SD. Statistical analyses where appropriate, were performed using one-way analysis variance ANOVA followed by the Dunnett's Multiple Comparison Test for unpaired data. A difference with a $P < 0.05$ was considered as statistically significant.

Results and discussion

Expression analyses and inducibility

The encoded peptides of each of the 13 predicted gallinacins contain the classic β-defensin six cysteine core, but the amino acid sequences are different. For this study Gals 4, 7, and 9 (Table 2), with calculated charges of +7, +7, and +4 at pH 7, hydrophobicity ratios of 31%, 24%, and 41%, respectively, were selected for further investigation.

Using systems optimised with the appropriate EST clones and RNA samples isolated from epithelial tissues and white blood cells of 38-day-old female chickens, Gal 7 expression was found to be ubiquitous. In contrast mRNA transcripts encoding Gals 4 and 9 were detectable only in a limited number of chicken epithelial tissues including the ovary (Gals 4 and 9), lung (Gal 9) and trachea (Gal 4) (Fig. 1A). Gallinacin in vivo gene expression patterns have previously been reported by two groups working independently. We show in support of others [13,14] that Gal 7 is expressed in the chicken small intestine, but in comparison to one report [13] were unable to confirm Gal 4 expression in the chicken (GI) tract, despite using a number of different PCR primers, GI tissues from birds of different ages and birds challenged orally with *Salmonella* (data not shown). In fact, in the

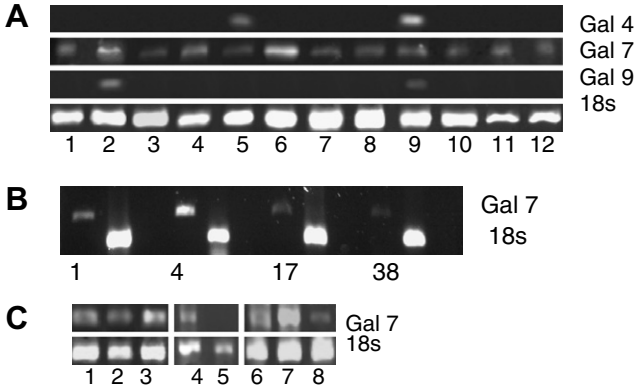

Fig. 1. (A) Gallinacin 4, 7, and 9 gene expression in chicken tissues. 1, Gizzard; 2, lung; 3, tongue; 4, rectum; 5, trachea; 6, skin; 7, white blood cells; 8, liver; 9, ovary; 10, small intestine; 11, kidney; 12, pancreas. (B) Gallinacin 7 gene expression in intestinal tissues excised from 1-, 4-, 17-, and 38-day-old chickens. 1, 1-day-old; 4, 4-day-old; 17, 17-day-old; 38, 38-day-old birds. (C) Gallinacin 7 expression in tissues excised from non-domesticated bird species. Small intestine (1), gizzard (2), and liver (3) of *Larus argentatus*; small intestine (4) and gizzard (5) of *Parus caeruleus*; small intestine (6), gizzard (7), and liver (8) of *Columba palumbus*.

Table 2
Multiple sequence alignment of gallinacins 4, 7, and 9 [13,14]

Peptide		Predicted Mass
Gal4	**MRILYLLLSVLFVVLQGVAG** QPYFSSPIHA--CRYQRGVCIPGPCRWPYYRVGSCGSGLK-SCCVRNRWA	5367
Gal7	**MKILCFFIVLLFVAVHGAVG** FSRSPRYHMQCGYRGTFCTPGKCPYGNAYLGLCRP---KYSCC--R-----WL	5029
Gal9	**MQILPLLFAVLLLMLRAEPGLSLA** RGLPQDCERRGGFCSHKSCPPGIGRIGL---CSK-EDF-CC---RSR-WYS	4750
	* * * * **	

Nomenclature follows that published by [13]. Signal sequences are in bold, asterisks indicate the six conserved cysteines and dashes are used to optimise the alignment. The predicted molecular masses of the mature gallinacins were calculated using the ExPASy Peptide Mass Programme.

38-day-old birds analysed in our study Gal 4 and 9 gene expression patterns were consistently localised. Why the expression patterns should differ between studies is at present unclear but factors influencing gene expression such as bird lineage, sex and infection status cannot be excluded.

Focussing on intestinal Gal 7 expression, RT-PCR analyses of RNAs prepared from small intestinal tissues excised from birds at different ages (1, 4, 17, and 38 days), suggested Gal 7 expression to be maximal within the first week post hatch and thereafter decline (Fig. 1B). Furthermore using the chicken specific PCR primers RT-PCR analyses of RNA excised from a number of epithelial tissues taken from non-domesticated bird species including *Parus caeruleus*, *Larus argentatus* and *Columba palambus* identified transcripts indicative of Gal 7 expression (Fig. 1C). However transcripts supportive of either Gal 4 or 9 expression were not detected (data not shown). The conservation of nucleotide sequence, and thus peptide, in environments where microbes can evolve rapidly suggests that the cationic gallinacin 7 peptide may have roles, in addition to antimicrobial activity, in the innate defences of the avian. Several human (h) β-defensins, including hβD-3, display chemoattractant activity for immature dendritic cells, monocytes and macrophages [19,20], thus it is possible that in the avian, Gal 7 may have a comparable role.

Salmonella are part of the natural intestinal flora of birds and challenging young 5-day-old chicks, used so as to reduce the effects of any factors associated with the activation of the acquired immune system [21], with *Salmonella* resulted in increased Gal 7 liver expression ($P < 0.05$) but no comparable increase in the small intestine (Fig. 2). These data suggest that the gallinacin genes can be either constitutively expressed or induced in response to microbial infection. In support, no increase in either Gal 1 or 2 expression was reported in the caecal tonsils of birds following an oral infection with *Salmonella* [22], and whereas the tracheal expression of Gal 3 was increased significantly in birds in response to *H. paragallinarum*, its expression in the tongue and oesophagus was not affected [11].

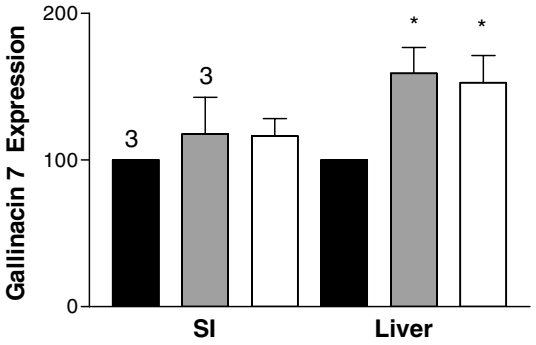

Fig. 2. Gallinacin 7 expression in chick tissues following oral infection with *Salmonella*. Semi-quantitaive RT-PCR analyses were performed on chick liver and small intestinal tissue samples 3 days after infection as described in materials and methods. The number of experimental observations in each group was four unless stated, and the values are means ± SEMs. $^{*}P < 0.05$ compared to the appropriate control. ■, control birds given PBS; ▨, birds given oral dose of 10^6 *S. enteriditis*; □, birds given oral dose of 10^6 *S. enterica serovar* Typhimurium SL 1344.

Anti-microbial activity

To investigate the antimicrobial activities of Gals 4, 7 and 9 against *Salmonella* the cDNAs encoding Gals 4, 7 and 9 were produced as His-tagged recombinant peptides using an Origami B::pLysS strain to promote disulphide bond formation [16]. SDS and Western analyses revealed bands of approximately 5 kDa, consistent with the predicted sizes of the gallinacins (Fig. 3A). The antimicrobial activities of the His-tagged recombinant peptides (antimicrobial potency has been shown previously not to be significantly altered by the presence of a His-tag [23]), and combinations thereof were tested against *Salmonella* species including *S. typhimurium pho*P with increased sensitivity to AMPs [24] and the wild type *S. enterica* serovar Typhimurium SL1344 and *S. enteriditis* strains. All three recombinant gallinacin peptides showed antimicrobial activity with the potency of the peptides in the order Gall $9 \geqslant 4 > 7$ (Fig. 3B). Assays conducted using combinations of peptides (final concentration 2 μM) were also suggestive of Gals 7 and 9, functioning synergistically to kill *S. enteriditis* (Fig. 3B-f).

The greater relative potencies of Gals 4 and 9 compared to Gal 7, may reflect a combination of their charge and hydrophobicity [25]. Antimicrobial peptides must first be attracted to bacterial surfaces and the obvious mechanism is through electrostatic bonding between the peptides and structures on the bacterial surface. *Salmonella* sp are known to resist cationic antimicrobial peptide activity by reducing the negative charge of their outer membranes through modification of the anionic membrane molecules including lipopolysaccharide (LPS) or lipid A, with positively charged substituents [26]. It is therefore possible that the reduced charge of Gal 9 (+4) compared to Gals 4 and 7 (+7), provides this peptide, at least in vitro, with a killing advantage against *Salmonella*. The actual killing mechanisms remain unknown although the hydrophobic nature of the peptides is indicative of a mechanism involving membrane permeabilisation and probably comparable to that reported for the ostricacins-1 and 2 [9]. However, at gallinacin concentrations associated with microbial killing less than 10% erythrocyte haemolysis was detected, indicating selectivity of the avian CAMPs for bacterial over mammalian cells (Fig. 4).

These data suggest that Gals 4, 7, and 9 function as antimicrobial agents and are an integral part of the avian host innate defence system.

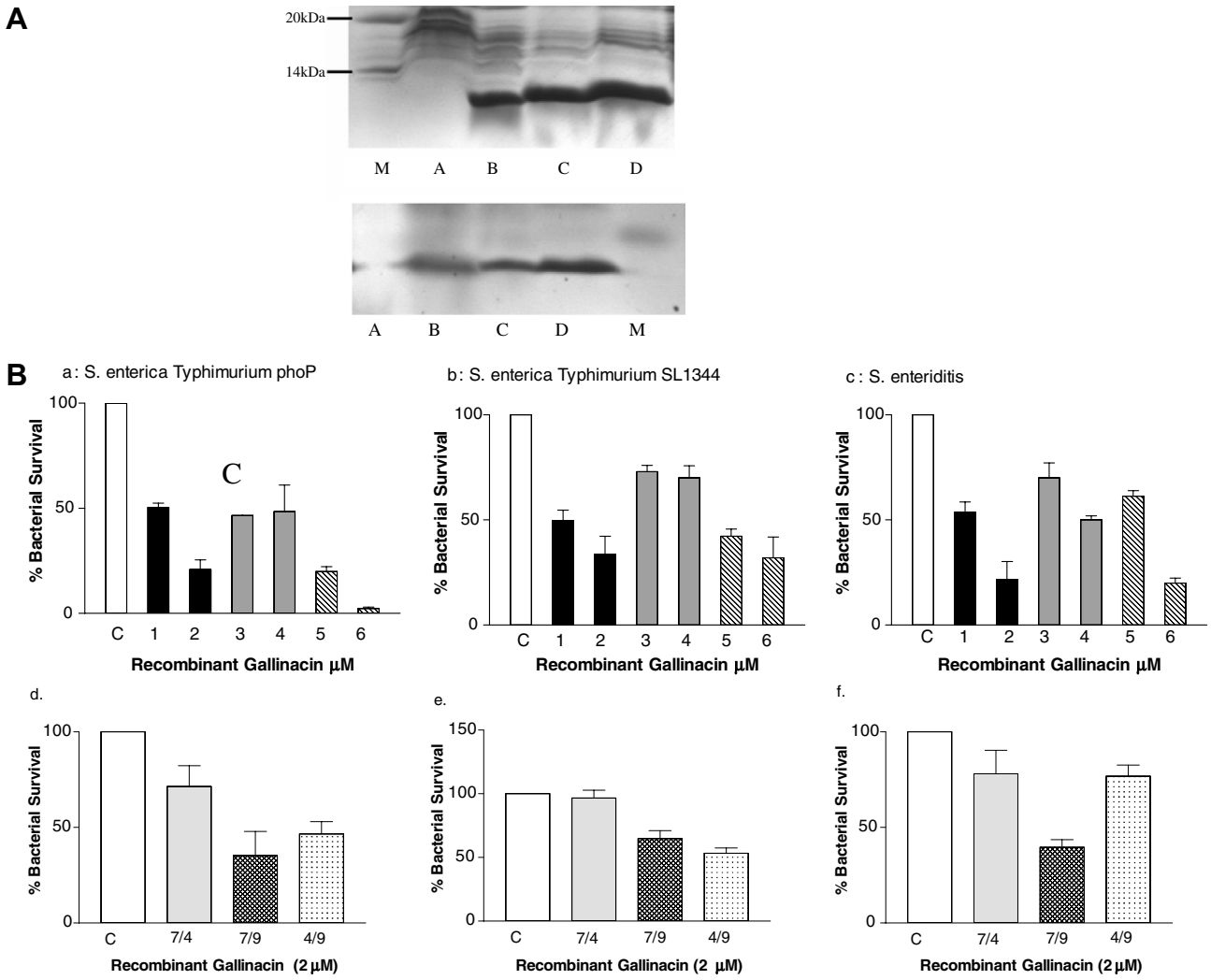

Fig. 3. Antimicrobial activities of the Gallinacins 4, 7, and 9. (A) SDS–PAGE (upper panel) of the His-tagged recombinant gallinacins 4 (B), 7 (C), and 9 (D) and His tag control (A); protein size marker (M). Western analysis (lower panel) of His tag peptide control (A), and the recombinant gallinacins 4, 7, and 9 using a 1:15,000 dilution of anti-polyhistidine antibody. (C) Gal 4 (lanes 1 and 2), 7 (lanes 3 and 4) and 9 (lanes 5 and 6) anti-microbial activities against *Salmonella* serovars. The number of colony forming units (CFU) surviving from a 3 h incubation with recombinant Gals 4, 7, and 9 are presented relative to that of the His-tag peptide control. Lanes 1, 3, and 5 reflect 2 μM recombinant peptide; lanes 2, 4, and 6 reflect 20 μM recombinant peptide. (D) Anti-microbial assays conducted using combinations of recombinant peptides at a concentration equivalent to 2 μM peptide. The minimum number of observations was 3 for each assay and values are presented as means ± SEMs.

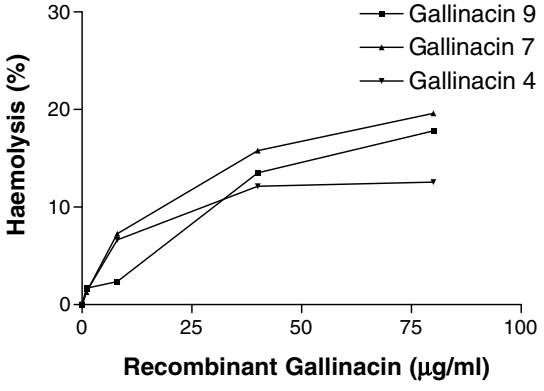

Fig. 4. Haemolytic activities of the Gallinacins. Haemolytic activities of Gals 4, 7, and 9 against chicken erythrocytes. Data points are means of $n = 3$ for each observation.

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