Introduction

*E. coli* K88 is a major pathogen that infects newborn and weaned piglets. This problem leads to great economic losses for the pork industry [1]. *E. coli* K88 is a Gram negative bacillus that expresses an adhesin-type lectin with specificity toward glycoproteins present in the intestine of piglets [2]. Additionally, this bacteria is toxin-producing and enterotoxigenic, triggering intestinal osmotic imbalance and severe diarrhea in susceptible animals [3]. Although there have been several studies aimed at the prevention of bacterial infection, when disease is established, the solution is antibiotic therapy.

The use of antimicrobials in animals raised for human consumption is controversial due to the constant increase in multidrug pathogen resistance to antibiotics [4]. Inadequate practices, such as the use of subtherapeutic antibiotic doses to promote animal growth or the use of high therapeutic doses, lead to increased bacterial resistance. Because antibiotics are the main tools for fighting infections, necessary measures for safe use should be taken. When farm animals have an infection, the parenteral route is the most commonly used route of administration for antibiotics. The concentrations obtained by intramuscular or intravenous routes of administration are much higher than an equivalent dose of a drug given orally [5]. However, intramuscular or intravenous routes of administration inevitably lead to systemic distribution of antibiotics, possible toxicity and accumulation in tissue [6-7]. Several studies agree that the localized administration of antimicrobials may lead to dose reduction and elimination of adverse side effects [7-8].

For gastrointestinal infections, the administration of poorly absorbable antibiotics (such as vancomycin, neomycin, colistin, nystatin and gentamicin) is an alternative treatment to produce localized activity and to reduce systemic toxicity [8]. Gentamicin is widely used to treat infections caused by *E. coli* K88 in pig farms [9]. In the last decade, gentamicin has been reported to cause less resistance than other antibiotics, including
spectinomycin, tetracycline, ampiollin, carbenicillin, neomycin and sulfamethoxazole [9]. Additionally, gentamicin is approved by the FDA for use in animals raised for human consumption[10]. For this reason, the efficient use of gentamicin is needed for E. coli K88 treatment. Oral administration of gentamicin is not effective, although it shows sensitivity in vitro [11]. The lack of activity in vivo could be related to poor distribution in the intestinal mucosa, which reduces both contact with and elimination of pathogens [12]. The rapid excretion of gentamicin through feces could also explain its low oral therapeutic effect.

An alternative delivery approach that may be useful for the distribution and maintenance of gentamicin in the gastrointestinal tract is the use of microspheres. Microspheres are transport systems based on solid polymers that allow for an effective control of drug release. In this regard, the controlled drug delivery systems have many benefits, for example, improved therapy by increasing efficacy and gastrointestinal transit time, convenient routes of oral administration and enhanced site-specific delivery to reduce unwanted adverse effects[13].

It has been shown that microparticles greater than or equal to 5 µm can contact and stay in the intestinal mucosa up to 24 h before elimination [14]. This behavior may allow the release and distribution of active substances in the intestinal space. In recent years, more than 100 biologically active compounds have been incorporated into albumin microspheres for many treatment applications [15]. Here, albumin microspheres were synthesized to encapsulate gentamicin. In vitro release of gentamicin was evaluated, and susceptibility tests with E. coli K88 were performed using encapsulated antibiotics in albumin microspheres.

**Materials and Methods**

**Materials**

Bovine serum albumin (BSA) fraction V, gentamicin sulfate, glutaraldehyde (25 wt %) and Span® 80 were purchased from Sigma-Aldrich, St Louis, Mo. USA. Culture medium was purchased from BD Difco™, MD, USA. Other chemicals used were analytical grade.

**Preparation of microspheres**

Gentamicin-albumin microspheres (GAM) were prepared according to the method proposed by Mathew et al. [16] and modified as follows. One milliliter of an aqueous solution with 20% BSA and 0.4% gentamicin sulfate was added to 30 mL of mineral oil containing 0.1% (wt/wt) Span 80® and stirred at 2000 rpm. After 1 h of continuous stirring (to obtain a homogeneous emulsion), 4.2, 8.2 and 16.8 mg/g of toluene-saturated glutaraldehyde were added to cross-link the G1, G2 and G3 microsphere formulations, respectively. Stirring continued for an additional 4 h, and then the microspheres obtained were recovered by centrifugation (5 min at 300 g). Finally, the microspheres were washed three times with hexane and dried at room temperature.

**Particle size distribution and morphology**

GAM particle size and distribution was measured using a Coulter Multisizer 3 (Beckman Coulter). Phosphate buffered saline (PBS) was used as a dispersing medium. Morphology was examined with scanning electron microscopy (SEM) using a Hitachi S-4800 Type II Ultra-High SEM. Samples were coated with conductive gold/palladium (60/40) prior to scanning at 5.0 kV [16].

**Yield, encapsulation efficiency and release efficiency of microspheres**

The obtained microspheres were weighed to determine yield using the following formula [17]:

Yield(%)=weight of microspheres obtained X 100 / weight of BSA+ weight of gentamicin

The encapsulation efficiency was determined by Fourier Transform Infrared (FT-IR) spectra according to Matkovic et al. [18] and Bunaciu et al. [19]. A Nicolet Protégé (System 460 E.S.P.) FT-IR spectrometer (Madison WI, USA) was used at a resolution of 0.5 cm⁻¹. Scanning was performed in the 4000-600 cm⁻¹ wavelength range. Quantitative analysis of gentamicin in the microspheres was performed with the FT-IR spectra of albumin:gentamicin (1:1, 2.5:1, 5:1 and 10:1). Samples were well mixed with potassium bromide (KBr), and KBr acted as an infrared-transparent matrix. Disks containing the mixture of albumin:gentamicin and KBr were compacted using a hydraulic press. Calibration curves were obtained by plotting the area under the peak at 1100-1400 cm⁻¹ vs. the concentration of gentamicin in the matrix. Encapsulation efficiency was obtained using the formula:

Ee%=amount of gentamicin found in microspheres x 100 / amount of gentamicin

Release efficiency was determined using a dialysis system [20]. Fifty milligrams of GAM dispersed in 5 ml of PBS (pH 7.2) was placed in a dialysis bag and incubated in 30 ml of the same buffer solution. The flask was subjected to sonication for 2 h. Subsequently, the microsphere suspension was placed in a shaking apparatus for three days. The gentamicin concentration in the external phase was quantified photometrically at 320 nm after derivatization with O-phthalaldehyde [21]. The derivatizing agent was prepared according to Fayle et al. [22]. Sixty milligrams of O-phthalaldehyde were solubilized in 30 ml of 0.1 M sodium borate with 200 µl of 2-mercaptoethanol and 20 ml of distilled water.

**In vitro release studies**

Twenty milligrams of GAM dispersed in 2 ml of PBS (pH 7.2) was placed in a dialysis bag and incubated in 20 ml of the same buffer solution. Similarly, the kinetics of release were evaluated in glycine-HCl buffer (pH 2), acetate buffer (pH 4) and PBS (pH 6). Dialysis studies were carried out at 37°C with continuous agitation.
At specific time intervals, 2 ml of external phase was taken to quantify the released gentamicin using a derivatizing agent (as described previously). Two milliliters of buffer was added to the external phase to compensate for the volume removed for analysis. In vitro release testing was repeated using GAM treated with trypsin and chymotrypsin under different conditions. Solutions were adjusted to 55 U for trypsin and 9.5 U for chymotrypsin in PBS (pH 7.4) according to Jensen et al. [23].

The release behavior of gentamicin from albumin microspheres was analyzed by a mass balance in the sphere, generating the first order equation:

\[ \frac{Dc}{dt} = -k_0c \]

where \( k_0 \) is the initial burst constant. Because the initial concentration in the particle is equal to the initial mass of drug loaded into microparticles per volume of microparticles, the solution becomes:

\[ \frac{m_t}{m} = 1 - e^{-kbt} \]

where \( m_t \) is the cumulative amount of drug released at time \( t \), \( m_0 \) is the initial amount of drug loaded in the microparticle, and \( m_t/m_0 \) is the fraction of drug release at time \( t \).

The first-order model was compared with the semi-empirical model introduced in 1983 by Peppas and co-workers to describe drug release from polymeric systems, commonly called the power law model [24].

\[ M_t / m = k^n \]

where \( k \) is a constant incorporating the geometrical and structural characteristics of the macromolecular network system and the drug and \( n \) is the diffusional exponent, indicative of the transport mechanism. This equation is valid for the first 60% of the fractional release. For spheres where \( n = 0.43 \), the transport mechanism is dominated by Fickian diffusion, while anomalous non-Fickian transport is described by \( 0.43 < n < 1 \). When \( n = 1 \), zero-order release is obtained [25-26]. The first-order model and the power law model contain unknown parameters that were adjusted to the experimental data by a nonlinear least-squares algorithm with the software MATLAB.

The criteria to choose the best model of release took into account the difference in the number of parameters between the first order model and the power law model and were based on the adjusted coefficient of determination [27].

\[ R^2_{\text{adjusted}} = 1 - \left( \frac{n_{dp}-1}{n_{dp}p} \right)(1-R^2) \]

where \( n_{dp} \) is the number of dissolution data points and \( p \) is the number of parameters in the model. The adjusted coefficient of determination is sensitive to the number of parameters in each model. The model that fits best will be represented by the highest adjusted coefficient of determination.

**Bacterial strain and growth conditions**

The \( E. coli \) K88 strain was donated by Dr. Carlos Eslava from the culture collection of Universidad Nacional Autonoma de Mexico and maintained as frozen stocks. Overnight cultures were grown in nutritive broth on a shaker at 37 °C. The bacterial suspensions were adjusted to 0.5 units of optical density at 600 nm (10^6 CFU/mL) before use.

**In vitro antimicrobial activity**

GAM suspensions were stirred constantly and serially diluted (from 0.1 mg/ml to 0.0015 µg/ml) in sterile nutritive broth and inoculated with 10 µL of \( E. coli \) K88 suspension (10^8 CFU/ml). Bacterial cultures were incubated at 37°C for 24 h, and the minimal inhibitory concentration (MIC) was determined as the lowest concentration without visible growth. MIC was confirmed with subcultures in trypticase soy agar plates.

**Sustained in vitro antibacterial activity**

Antibacterial activity was determined as described by Egbaria et al.[28] and Wang et al. [29] with modifications. GAM (1 mg/mL) was suspended in nutrient broth (as release medium) with constant shaking at 37°C. The media was removed every hour as follows: suspensions of microspheres were centrifuged, the supernatant was placed in sterile vials and the pellet was resuspended in new sterile nutrient broth. The process was repeated every hour until 12 samples were collected. All recovered supernatants (nutrient broth/gentamicin-released) were inoculated with 10 µL of \( E. coli \) K88 suspension (10^8 CFU/ml) and incubated as described previously.

**Results and Discussion**

**Microsphere characteristics**

GAM were prepared using the strategy of emulsion polymerization chemistry [16]. Glutaraldehyde-saturated toluene (GST) was used to promote polymerization on the surface of the GAM [16]. The G1, G2 and G3 treatments included glutaraldehyde concentrations of 4.2, 8.4 and 12.6 mg/g, respectively. The G1, G2, and G3 treatments presented free spherical particles, as determined by SEM studies (Figure 1). A tendency to form indentations with increasing amounts of GST was observed (Figure 1D). Indentations could be attributed to collisions or interactions between microspheres during synthesis when glutaraldehyde saturated with toluene was added one hour after starting emulsion, because at this time, the microspheres lost water and the surfactant was removed, followed by the interaction of the microspheres [30] Toluene works as a surfactant remover and also decreases the viscosity of the continuous phase. Smaller particles set faster and could collide with the surface of larger particles that harden more slowly.
The average particle size for each treatment varied from 10.5-12.3 μm (Table 1). The particles were within the size range of those that are reported to remain in the intestinal lumen [14]. This retention is important to allow the release of antibiotics in areas typically infected by pathogens [31]. The yield for all treatments (G1, G2 and G3) was higher than 93%, and the encapsulation efficiency of gentamicin ranged from 87.5% to 97.3% (Table 1). However, the release efficiency of gentamicin appeared to be inversely affected by the amount of glutaraldehyde used for cross-linking the microspheres (Table 1). These results could be ascribed to glutaraldehyde cross-linking both albumin and gentamicin amino groups [32].

Gentamicin quantitation

GAM were studied using infrared analysis to detect and quantify gentamicin in albumin microspheres. FT-IR is a common technique used to identify drugs; however, it is not widely used for quantification purposes. As expected, gentamicin and albumin showed different infrared spectra: for gentamicin, major absorbance peaks were at 619 and 1100-1400 cm⁻¹, while for the albumin amide group, absorption occurred at 1651-1653 and 1574-1548 cm⁻¹. The GAMs' spectra showed both albumin and gentamicin signals (Figure 2) that allowed for their rapid identification. To quantify gentamicin, a standard calibration curve was prepared; using the equation y = 0.0468x + 0.2552, the coefficient of determination obtained (R²) was 0.995. The G1, G2 and G3 treatments presented gentamicin encapsulation efficiencies of 97.3±2, 92.2±2 and 87±0 (%), respectively (Table 1). There was a decrease of gentamicin content with increasing concentrations of cross-linking agent. This behavior is in agreement with reports used to identify drugs; however, it is not widely used for quantification purposes.

In vitro release studies

Gentamicin release was measured using a dynamic dialysis system. In general, we found that gentamicin is released in a one-step profile, as shown in Figure 3. During the first four hours, 69%, 72% and 92% of the gentamicin was released from G1, G2, and G3, respectively (Figure 3A). It is important to note that during diarrhea in pigs, intestinal transit from food intake to gastric emptying may take from 3 to 4 h [34]. Because of this, it is critical that therapeutic microspheres release antibiotic during the first four to six hours to counteract the colibacillosis present from the jejunum to the rectum.

In these studies, the antibiotic liberation behavior was not affected by the amount of glutaraldehyde used. However, the amount of gentamicin released (release efficiency) was inversely proportional to the amount of glutaraldehyde present, as described previously. Similar antibiotic behavior was reported for vancomycin released from albumin microspheres [35]. Under acidic conditions (pH 2 and 4), the release of gentamicin was faster than at pH 6 (Figure 3B). Most of the gentamicin was released from the albumin microspheres (G1, G2 and G3 formulations) during the first hour, probably because gentamicin is highly soluble at acidic pH, which allows for a rapid diffusion from the microspheres to the medium. This result is not convenient for an intestinal carrier system, which should be able to retain a drug in the acidic pH of the stomach environment. However, the problem could be solved by encapsulating the microspheres in gelatin capsules [36-37]. Furthermore, as shown in figure 3C, the release of gentamicin from G2 was not affected by protease treatment.

The release of gentamicin from GAM at pH 7.2 was analyzed using a first order model and compared to the power law model. Figure 4 A, B and C show the matching between the first order model and the power law model along with the experimental data for G1, G2 and G3, respectively. In the first order model, we found that the initial burst constant increases as the degree of cross-linkage in the microparticles preparation increases. The diffusional exponent obtained from the power law model for the three release studies was below the value of 0.43 expected for Fickian release behavior in spherical geometries. This result could be attributed to particle size distribution affecting the release kinetics. In comparison to the release behavior from a monodisperse sample, the presence of a particle size distribution causes a substantial acceleration in transport at early times and a marked retardation in transport at longer times[25]. Comparison between the adjusted coefficients of determination from both models indicates that gentamicin release from GAM could be better described by a first order equation than by a power law model. The parameters of both release models and the fitting with gentamicin release from GAM at pH 7.2 are listed in Table 2.

In vitro antimicrobial activity

GAM antibacterial activity against E. coli K88 was tested using the broth dilution technique (Table 3). The MIC for the G1, G2 and G3 formulations was 3.12, 6.25 and 6.25 μg/ml, respectively. The
Figure 1. Scanning electron micrograph of gentamicin albumin-microspheres. (A) G1; (B) G2; (C) G3 and (D) close up of G3

Figure 2. FT-IR of BSA, gentamicin and gentamicin albumin-microspheres.

Table 1. Parameters and characteristics of gentamicin albumin-microspheres.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Glutaraldehyde added (mg)</th>
<th>Yield (%)</th>
<th>Encapsulation efficiency (%)*</th>
<th>Release Efficiency (%)</th>
<th>Mean particle size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>0.28</td>
<td>94 ± 4.0</td>
<td>97.3 ± 2</td>
<td>93.3 ± 3.3</td>
<td>10.5 ± 0.4</td>
</tr>
<tr>
<td>G2</td>
<td>0.56</td>
<td>97 ± 1.5</td>
<td>92.2 ± 2</td>
<td>62.5 ± 5.0</td>
<td>12.0 ± 0.7</td>
</tr>
<tr>
<td>G3</td>
<td>0.84</td>
<td>93 ± 4.1</td>
<td>87.5 ± 0</td>
<td>43.6 ± 6.5</td>
<td>12.3 ± 0.6</td>
</tr>
</tbody>
</table>

* Determined by FT-IR
Figure 3. *In vitro* release profile of gentamicin from microspheres. (A) G1, G2, G3 and gentamicin in PBS (pH 7.2). (B) G2 in glycine-HCL, acetate and phosphate saline buffer at pH 2, 4 and 6, respectively. (C) G2 in phosphate buffered saline (pH 7.4) with trypsin and chymotrypsin.

Figure 4. Models and experimental data matching gentamicin release from microspheres. (A) G1, (B) G2, and (C) G3. The points represent the experimental data; the solid line represents the first order release model, and the dashed line represents the power law model.
Table 2. Parameters of gentamicin release from albumin microspheres in PBS, pH 7.2.

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameter</th>
<th>Description</th>
<th>Unit</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power law</td>
<td>$k$</td>
<td>Characteristics of the macromolecular network system and the drug</td>
<td>hr$^{-1}$</td>
<td>0.3996</td>
<td>0.4713</td>
<td>0.5629</td>
</tr>
<tr>
<td></td>
<td>$n$</td>
<td>Diffusional exponent</td>
<td>-</td>
<td>0.3204</td>
<td>0.2624</td>
<td>0.2195</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>Coefficient of determination</td>
<td>-</td>
<td>0.915</td>
<td>0.950</td>
<td>0.882</td>
</tr>
<tr>
<td></td>
<td>$n_{dp}$</td>
<td>Number of dissolution data points</td>
<td>-</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>$R^2_{adjusted}$</td>
<td>Adjusted coefficient of determination</td>
<td>-</td>
<td>0.898</td>
<td>0.940</td>
<td>0.858</td>
</tr>
<tr>
<td>First order</td>
<td>$k_b$</td>
<td>Burst constant</td>
<td>hr$^{-1}$</td>
<td>0.2867</td>
<td>0.3592</td>
<td>0.5058</td>
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<tr>
<td></td>
<td>$R^2$</td>
<td>Coefficient of determination</td>
<td>-</td>
<td>0.989</td>
<td>0.988</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td>$n_{dp}$</td>
<td>Number of dissolution data points</td>
<td>-</td>
<td>7</td>
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</tr>
<tr>
<td></td>
<td>$R^2_{adjusted}$</td>
<td>Adjusted coefficient of determination</td>
<td>-</td>
<td>0.989</td>
<td>0.988</td>
<td>0.994</td>
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</table>

Table 3. Minimal inhibitory concentration (MIC) obtained for gentamicin albumin-microspheres.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MIC (µg/ml)</th>
<th>GAM</th>
<th>Estimated [gentamicin] in GAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>3.125</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>6.25</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>6.25</td>
<td>0.44</td>
<td></td>
</tr>
</tbody>
</table>

MIC of free gentamicin = 0.31 (µg/ml)

actual content of gentamicin (expressed as released efficiency, Table 1) was estimated to be 0.47, 0.63 and 0.44 mg/ml for G1, G2 and G3, respectively. These values are close to the MIC obtained for free gentamicin (0.31 g/ml). Additionally, the MIC of gentamicin against *E. coli* K88 found in this experiment is similar to the one reported for *E. coli* ATCC 25922 (0.5 µg/ml) [38]. Also, our data indicate that the encapsulation process by W/O emulsion does not affect the antibacterial activity of gentamicin. In contrast, Haswani et al. [20] reported a decrease in the antimicrobial activity of gentamicin encapsulated in albumin microspheres produced by the spray-dried method.

Sustained in vitro antibacterial activity

The antibacterial activity of gentamicin encapsulated in microspheres (formulation G2) against *E. coli* K88 was determined every hour for 12 h (1 mg of GAM was used). The released gentamicin maintained its antibacterial activity during the entire 12 h of the experiment. According to the release kinetics shown in figure 3A, the amount of gentamicin released from 1 mg of G2 at 12 h remained in the released medium at an approximate concentration of 1.6 µg/ml. This amount of gentamicin was higher than the MIC of G2, as shown in Table 3.
Conclusions

The aim of this work is to determine the future use of GAM for oral transportation of gentamicin as a specific treatment for swine colibacillosis. Gentamicin-loaded albumin microspheres were obtained by emulsion and cross-linked with glutaraldehyde. The use of this cross-linking agent did not modify the release profile but did decrease the release efficiency. Chymotrypsin and trypsin activity did not affect the GAM’s properties, but acidic conditions increased drug release. The release of gentamicin from GAM could be described by a first order equation. Gentamicin from the microspheres showed antibacterial activity against E. coli K88. These results suggest that microsphere-gentamicin is a potential drug carrier and could be used for therapy against E. coli K88 infection.

Abbreviations and nomenclature

Gentamicin-albumin microspheres (GAM), water/oil (W/O), Escherichia coli K88 (E. coli K88), Bovine serum albumin (BSA), Phosphate buffered saline (PBS), Fourier Transform Infrared (FT-IR), minimal inhibitory concentration (MIC), Glutaraldehyde-saturated toluene (GST).

Authors' contributions

ASS. As first author, my contribution to this paper was to formulate and characterize the gentamicin-loaded albumin microspheres as a potential drug carrier for treatment E.coli/K88 infections, and to apply most of the techniques indicated in the present work. Also, to gather and analyze results from other collaborators.

GRCM. I am part of the research group and my contribution for the Project is related to provide information and critical review of concepts expressed in this paper.

LVM. I am the leader of the group and the Project is under my responsibility. I supervised the design of experiments and original methodologies applied to evaluate the antimicrobial activity of gentamicin-loaded albumin microspheres. As well as analysis and critical review of concepts expressed in this paper.

ALA. My contribution to this work included the design and analysis of data which based on mass balance of gentamicin-loaded albumin microspheres could generate an equation capable of explaining the release of antibiotic from microspheres.

RZG. My contribution to this work included the design and analysis of microsphere-gentamicin is a potential drug carrier and could be used for therapy against E. coli K88 infection.

Conflict of Interest

There is no conflict of Interest

Acknowledgements

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