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Short communication

Cloxacillin nanostructured formulation for the treatment of bovine keratoconjunctivitis

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ABSTRACT

Infectious bovine keratoconjunctivitis (IBK) is a widespread, contagious ocular disease that affects cattle, especially dairy breeds. The disease is caused by Gram-negative bacteria mainly *Moraxella bovis,* and its treatment consists of parenteral or topic antibiotic therapy. The topic treatment approach is used more commonly in lactating cows, to avoid milk disposal. However, treatment failures are common, because the antibiotic is removed during lacrimation. This study aimed to evaluate the susceptibility of commercial cloxacillin and evaluate the efficacy of nanostructured cloxacillin in clinical cases of IBK by *Moraxella*. The minimum inhibitory concentration (MIC) of nanoparticle cloxacillin nanocoated, the nanoparticle without the antibiotic and the commercial cloxacillin were determined *in vitro* with field samples of *Moraxella ovis* (5) and *Moraxella bovis* (5). The efficiency of nanoparticles was tested in three cows naturally infected that were treated with 1.0 mL (with 0.32 mg of nanostructured cloxacillin) for the ocular route. *Moraxella bovis* was isolated and identified by biochemical and molecular methods before the treatment. The animals were treated every 12 h for six days. The cure was considered by the absence of clinical symptoms and bacteria after treatment. The mucoadhesive nanoparticle-based formulation promoted clinical cure with a low number of doses of antibiotics, probably due to the maintenance of the MIC in the ocular mucosa for longer due to the mucoadhesive characteristics of the nanoparticle. The results indicate that the use of nanocoated cloxacillin is possible to control infectious bovine keratoconjunctivitis.

Infectious bovine keratoconjunctivitis is the most common ocular disease of cattle and affects herds in different regions of the world. The primary etiological agent of IBK is *Moraxella bovis*. The first report of *M. bovis* and IBK to cause the disease in cattle was done by Jones & Little (1923). Although both dairy and beef breeds of all ages are susceptible to IBK, calves and confined animals are most affected. The infection is characterized by corneal ulceration which can lead to corneal scarring (Angelos & Ball, 2007). The disease rarely results in death but can cause a significant reduction in productivity in infected animals (Angelos & Ball, 2007; Postma, Ce, & Minatel, 2008).

Generally, *M. bovis* infections are treated topically using formulations containing antibiotics, mainly when clinical signs are observed. The antibiotic benzathine cloxacillin is known to be effective for the treatment of *M. bovis* infections, and its topic administration has been widely recommended (Angelos, Ball, Byrne , 2015). Ophthalmic drugs containing benzathine cloxacillin are commercially available in some countries, for example, in the United Kingdom (George et al., 1989), but they are not usually used in Brazil. Recurrences of the disease after treatment have been reported, especially as a result of treatments using ineffective antibiotics or carried out incorrectly (George et al., 1989; Bar, Gümüfisoy, & Öztürk, 2006; Connor, Shen, Wang, Opriessnig, 2012)

Nano-sized formulations can be used as ocular drug delivery systems, with the advantages of enhancing the absorption of the therapeutic drugs, improving bioavailability, reduced systemic side effects, and sustaining intraocular drug levels (Stella et al., 2012; Wadhwa, Paliwal, Paliwal, & Vyas, 2010). Therefore, the use of polymeric nanoparticles can be considered a winning strategy for the topical

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treatment of ocular diseases. In this way, we have developed a formulation containing nanocapsules that can promote a greater ability of adherence of cloxacillin benzathine to the ocular surface, by electrostatic interactions, since that chitosan is a cationic polymer with positive surface and binds to negative structures like mucin of bovine eyes surface. In this study, we compared the MIC of cloxacillin benzathine incorporated into mucoadhesive nanoparticles with a cloxacillin-containing formulation commercially available. Besides, we also evaluate the clinical use of nanocapsuled-cloxacillin for the treatment of clinical IBK in dairy cows.

An outbreak of clinical keratoconjunctivitis occurred in a dairy herd with approximately 70 lactating Holstein cows, located in Minas Gerais state. The ocular clinical signs were examined daily and animals presenting corneal ulcers were selected for the study. Protective garments were worn during the tests and were rinsed in a 1% chlorhexidine solution after examination or treatment of each animal.

Ocular secretions of affected animals were obtained by wiping the inferior conjunctival cul-de-sac using cotton-tipped swabs. The harvested material was inoculated immediately into plates containing 5% ovine blood agar. The blood agar plates were kept chilled on ice until they were transported to the laboratory. Subsequently, the plates were streaked and incubated for 48 h. Colonies that resembled *M. bovis* were picked, subculture until pure, and identified using morphological characteristics, staining method such as Gram, and biochemical criteria as the production of oxidase, catalase and KNO_3 (Macfaddin, 2000). Isolates identified as *M. bovis* were stored in a milk medium at −80 °C. The biochemical identification was confirmed by polymerase chain reaction (PCR) coupled with analysis of restriction enzyme digestion of amplified DNA with *RsaI* (Angelos & Ball, 2007).

After identification by molecular and biochemical evidence, the MIC of nanoparticle cloxacillin nanocoated, the nanoparticle without the antibiotic (negative control) and the commercial cloxacillin were determined *in vitro* with field samples of *M. bovis* (CLSI, 2006). This field strain was originated from the outbreak on the farm of Embrapa Dairy Cattle, Southeast of country, and other strains such as *M. bovis* (5 strains) and *M. ovis* (5 strains) originated from Rio Grande do Sul state, Southern Brazil. *Staphylococcus aureus* (ATCC 29213) was used as a quality control test, and each laboratory tests were performed in duplicate.

Poly(ε-caprolactone) coated chitosan nanocapsules (PCL-CS NC) containing cloxacillin were prepared by interfacial deposition of the preformed polymer, followed by evaporation of the solvent, according to a methodology described previously (Mosqueira et al., 2011; Slatter et al., 1982). The average size and polydispersity index (PDI) of the particles were determined by photon correlation spectroscopy at 20 °C in a Nanosizer N5Plus Analyzer Beckmann Coulter (Fullerton, USA), while the zeta potential was determined by laser Doppler anemometry in a Zetasizer HS3000 (Malvern Instruments, Malvern, UK) (Reis & Neufeld, 2006). A sterile suspension of benzathine cloxacillin was used, and each 0.32 mg of nanostructured cloxacillin was suspended in approximately 1 ml of a sterile aqueous suspension, and the dose for each eye was suspended in a separate syringe.

Before treatment, the swab was used to collect material from all animals to identify the bacteria present in the outbreak. Three cows naturally infected were treated with 1.0 mL (with 0.32 mg of nanostructured cloxacillin) for an ocular route for six days at intervals of 12 h, after intense lacrimation and the presence of an ulcer with 1 cm^2. After treatment, the clinical evaluation was performed based on the healing of ulcers and absence of tearing. The PCL-CS NC used as topic treatment had an average diameter of 322 nm and a low polidispersal with 0.088, which features single mode dispersion. The zeta potential was estimated at −28 mV, indicating that the particles can be stable in aqueous suspension by electrostatic repulsion, once the magnitude of zeta potential is very close to 30 mV (Mosqueira et al., 2011). The MICs for cloxacillin and nanocoated cloxacillin, as well as the negative control and the nanoparticles without antibiotic, are presented in Table 1. In the negative control, bacterial growth was observed, and the MIC's for cloxacillin and nanocoated cloxacillin were similar for the two formulations. *Moraxella bovis* was identified as the etiological agent for IBK in this study.

The nanostructured cloxacillin is encapsulated inside the nanoparticles, with less contact with the Moraxella *in vitro* tests. The MIC results from commercial cloxacillin and nanocoated cloxacillin were similar, showing that the encapsulation process did not cause degradation of cloxacillin. In the MIC test, the presence of biological fluids does not occur as well as, compartments or molecules that can reproduce the bioavailability of the antibiotic at the animal organism and, thus, it is efficient against the pathogen.

The affected Holstein cows had excessive tearing, photophobia, corneal opacification, and edema. Although calves are generally more susceptible than older cattle (Webber & Selby, 1981; Slatter, Edwards, Hawkins, Wilcox, 1982), adults can be severely more affected when the herd has not previously exposed (Brown, Brightman, Fenwick, & Rider, 1998). Despite the fact that all breeds may be affected, *Bos taurus* is more susceptible than *Bos indicus* breeds (Webber & Selby, 1981; Frisch, 2010).

This is the first study conducted in Brazil with a nanostructured antibiotic (cloxacillin) for the treatment of clinical IBK, as a result of a natural outbreak on a dairy herd. Infectious bovine keratoconjunctivitis outbreaks are relatively frequent in dairy cattle in Brazil (Paolichi, Leturia, Gil, 2004), mainly in the southern region of the country. Since it is the first treatment of natural cases of IBK with nanostructured molecules, the cloxacillin nanostructured showed a curative effect when applied locally in the eyes of the affected animals every 12 h, with a clinical and microbiological cure after six days of treatment with nanocapsuled-cloxacillin. It is possible to infer that nanocapsuled-cloxacillin had better adherence to ocular mucosa, making the treatment easier (fewer treatments per day) and lowering drug removal for the lachrymal secretions. The lower possibility of occurrence of residues in milk and meat from treated animals, the longer contact time with the bacteria and the better effectiveness of the treatments of clinical cases in ruminants, decreases the selection pressure on the antimicrobial-resistant bacterial population. Thus, it increases the useful antimicrobial life.

Chitosan is a cationic polymer (nanocoated cloxacillin) that gets positively charged with nanoparticles that bind to negatively charged structures, by electrostatic interactions. The eyes' surfaces of epithelial cells has a membrane-spanning mucin to form a glycocalyx (Rolando and Valente, 2007), bovine mucin has a negative charge, due to the presence of sialic acid residues in the oligosaccharide units (Lu, Kostanski, Ketelson, Meadows, & Pelton, 2005), because its carboxyl group tends to dissociate a proton at physiological pH. Similarly, the glycosaminoglycans are rich in sialic acid, and it disperses in the cornea and sclera (Trier & Ribel-madsen, 2004). Ulcerative keratitis exposes extracellular matrix that is rich in glycosaminoglycans (Squirrell, Winfield, & Amos, 1999). It occurs in consequence of hyaluronic acid, and chondroitin sulfates have a negative charge. This situation allows a longer permanence of particles in the eye, especially in animals clinically affected.

The main difficulty in the therapy of ocular infection is the topical treatment since several applications are required per day, to maintain the therapeutic levels of antimicrobial. In herds that topical administration is hard to do, the intramuscular administration of antimicrobials, such as oxytetracycline, is the alternative. However, this approach proves extremely costly, mainly for lactating animals because of milk disposal. The use of nanocarrier's mucoadhesive may be an alternative to overcome the drawbacks of the use of both the parenteral therapy as the topical ocular administration, promoting the sustained release of drug at ocular mucosa, keeping the concentration of the antimicrobial nanostructured higher, reducing the number of applications, and reducing milk disposal in cases of parenteral therapy.

The MIC results of nanostructured and conventional cloxacillin were

Table 1
Susceptibility of *Moraxella bovis, Moraxella ovis* and *Staphylococcus aureus* (ATCC 29213) to commercial cloxacillin (CLOX) and nanocoated cloxacillin (NPCLOX).

		Minimum inhibitory concentration μg/mL		
Strain	Origin (State)	CLOX	NPCLOX	Negative control
M. bovis 01	Minas Gerais	1	1	> 64
M. bovis 02	Rio Grande do Sul	0,5	0,5	> 64
M. bovis 03	Rio Grande do Sul	4	4	> 64
M. bovis 04	Rio Grande do Sul	2	2	> 64
M. bovis 05	Rio Grande do Sul	1	1	> 64
S. aureus ATCC 29213		0,5	0,5	> 64
M. ovis 02	Rio Grande do Sul	0,5	0,5	> 64
M. ovis 03	Rio Grande do Sul	1	1	> 64
M. ovis 04	Rio Grande do Sul	1	1	> 64
M. ovis 05	Rio Grande do Sul	0,5	0,5	> 64

similar; nanocoated-cloxacillin can be used in ophthalmic formulations with similar concentrations to commercial formulations. Preliminary results indicate the promising use of cloxacillin nanostructured to infectious bovine keratoconjunctivitis. However, to confirm these findings and provide security for their use, the number of treated animals should be increased.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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