# BRIEF COMMUNICATION: *In vitro* evaluation of the antimicrobial effects of chitosan against bacteria involved in ovine footrot

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## Introduction

Virulent footrot of ovines is caused by a mixed infection of *Dichelobacter nodosus* and *Fusobacterium necrophorum*; anaerobic, non-spore forming Gram negative bacilli. Footrot has a significant economic impact on the farming industry within New Zealand (Hickford et al. 2005).

When the interdigital skin of the ovine foot is damaged, or wet for a prolonged period, it may be infected by *F. necrophorum*, a member of the gut flora, which causes ovine interdigital dermatitis. The skin becomes red and swollen and bacterial toxin production results in necrosis in the upper layers of the skin enabling *D. nodosus* to establish if the organism is present in the flock or the environment. *D. nodosus* can survive for seven to 10 days on pasture and six weeks on hoof horn clippings. Virulent footrot causes degrees of lameness as *D. nodosus* digests the living tissue/dermis using collagen as its substrate. This eventually leads to the separation of the hoof horn from the underlying tissues. The associated trauma results in the reduction of animal weight, fertility, and wool growth (Kennan et al. 2011).

Current treatments for footrot are either topical or parenteral. Current topical treatment requires careful hoof paring to remove all under run horn so as to expose necrotic tissue. Bactericidal solutions are then applied by aerosol spray or by foot bathing in 10% zinc sulphate, 10% copper sulphate, or 5% formaldehyde. Parenteral treatment consists of injections of penicillin and streptomycin. Vaccinations are available but are expensive.

Chitosan, a linear polysaccharide composed of randomly distributed β- (1-4) linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), produced commercially by deacetylation of chitin (the exoskeleton of crustaceans such as crabs and shrimps), has well described antibacterial and antifungal properties (Vinsova & Vavrikova 2008).

The results described in this paper are from a pilot study designed to investigate the viability and efficacy of chitosan as an alternative and sustainable topical footrot treatment.

## Materials and methods

### *Chitosan*

Four chitosan derivatives were prepared from Alaska snow crab (commercial grade; Waseta, China; Molecular weight 890 kDa) using different concentrations of acetic acid as described by Ghosh & Ali (2012). Preparations were labeled A to D in increasing order of acetic acid concentration and then formulated to contain between 1% and 5% chitosan , labeled as 1 to 5 and the two codes combined to create a sample label.

### *Bacterial strains*

Bacterial strains of *Dichelobacter nodusus* (AgResearch Wallaceville) and *Fusobacterium necrophorum* (NZRM 1109 (ESR, Porirua)) Type A, were grown in Brain Heart Infusion Broth (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) plus 1% yeast extract (Difco, Becton Dickinson, Franklin Lakes, NJ, USA), and 0.05% cysteine (Sigma, St. Louis, MO, USA) for 24–48 hours in an AnaeroPack System™ (Mitsubishi Gas Chemical America Inc., New York, NY, USA) at 37°C.

### *Minimum inhibitory concentration assays*

The microplate method described by Tayel et al. (2010) was used to determine the minimum inhibitory concentrations (MIC) of chitosan samples against *D. nodusus* and *F. necrophorum*. Three replicate experiments were conducted at 37°C and two at 25°C.

Twenty microlitres of bacterial culture (approximately $10^8$ colony forming units/mL) were added to wells in a 96 well microplate. Chitosan dissolved in 1% acetic acid (100 µL) was placed in the first well and serial dilutions made resulting in a final concentration range from 0.5 mg/mL down to 0.0078 mg/mL. Chitosan free 1% acetic acid was used as a blank control. The therapeutic antibiotic streptomycin sulphate (Sigma, St. Louis, MO, USA) was used as a positive control at concentrations ranging from 1 mg/mL to 0.015 mg/mL. Microplates were incubated overnight at 25°C or 37°C in an AnaeroPack System™. As an indicator of bacterial growth 20 µL of p-iodonitro-tetrazolium violet (INT) (USB Corporation, Cleveland, Ohio, USA) dissolved in water at a concentration of 0.4% w/v was added to

**Table 1** Minimal inhibitory concentrations of four chitosan derivatives extracted using different concentrations of acetic acid (A – D) with each preparation containing between 1% and 5% chitosan (1 – 5) against *Dichelobacter nodosus* and *Fusobacterium necrophorum* grown at 37°C (mean of three experiments) or 25°C (mean of two experiments). Streptomycin sulphate was used as the control.

| Chitosan derivative | Minimal inhibitory concentrations (mg/L) | | | |
|---|---|---|---|---|
| | 37°C | | 25°C | |
| | *Dichelobacter nodusus* | *Fusobacterium necrophorum* | *Dichelobacter nodusus* | *Fusobacterium necrophorum* |
| A-1 | 21 | 208 | 7.8 | 125 |
| A-2 | 34 | 167 | 7.8 | 125 |
| A-3 | 34 | 208 | 7.8 | 125 |
| A-4 | 91 | 167 | 7.8 | 250 |
| A-5 | 94 | 208 | 7.8 | 500 |
| B-1 | 10 | 208 | 7.8 | 250 |
| B-2 | 13 | 208 | 7.8 | 125 |
| B-3 | 10 | 292 | 7.8 | 125 |
| B-4 | 13 | 417 | 7.8 | 250 |
| B-5 | 10 | 417 | 7.8 | 250 |
| C-1 | 16 | 208 | 62.5 | 250 |
| C-2 | 10 | 292 | 7.8 | 250 |
| C-3 | 13 | 250 | 7.8 | 250 |
| C-4 | 13 | 333 | 7.8 | 250 |
| C-5 | 60 | 500 | 156 | 250 |
| D-1 | 13 | 292 | 7.8 | 250 |
| D-2 | 10 | 292 | 7.8 | 250 |
| D-3 | 10 | 417 | 7.8 | 250 |
| D-4 | 10 | 417 | 7.8 | 250 |
| D-5 | 10 | 417 | 7.8 | 500 |
| Streptomycin | 62 | 144 | 62.5 | 250 |

the wells and incubated at 37°C for 30 minutes. The colourless salt was reduced to a red coloured formazan product by biologically active organisms. MIC values were recorded by eye as the lowest concentration of chitosan that inhibits bacterial growth. To determine if the effect of the chitosan on the organism was bactericidal, 50 µL from each well was spread on agar and incubated as previously described.

### *Bacterial survival experiments*

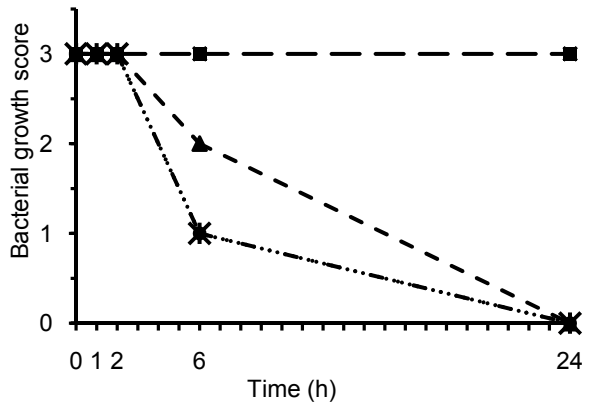
Chitosan fraction A-1 was incubated with *Dichelobacter nodusus* at concentrations of 625 µg and 1,250 µg/mL of Brain Heart Infusion Broth plus 1%, yeast extract and 0.05% cysteine for 48 hours in an AnaeroPack System™ at 37°C. Streptomycin sulphate at concentrations between 30 mg/L and 1,020 mg/L were run as controls and values obtained with 128 mg/L were used to plot control data. Samples were taken at 30 minutes, 1, 2, 6, 24 and 48 hours and bacterial growth measured by subculturing 10µL aliquots of broth onto agar plates and incubating as described. Agar plates were ranked as: 0 = No growth, 1 = Light growth, 2 = Moderate growth and 3 = Heavy growth. Experiments were performed in duplicate.

## Results

### *Antimicrobial activity of chitosan derivatives*

The antimicrobial activity, in terms of MIC, of the twenty chitosan derivatives against *D. nodusus* and *F. necrophorum* are shown in Table 1. All the preparations showed some antimicrobial activity against the two organisms at both temperatures. Results were the same for all replicate experiments. Some preparations had antimicrobial activity similar to, or greater than, the therapeutic antibiotic used as a control at both temperatures against *D. nodusus* (Table 1). All the fractions except C-1 and C-5 had greater efficacy against *D. nodusus* at 25°C than at 37°C. Antimicrobial activity against *F. necrophorum* was not as marked as that against *D. nodusus*. In general, the MICs were similar at both temperatures with some exceptions. The therapeutic antibiotic streptomycin sulphate had a higher MIC at 25°C than at 37°C for *F. necrophorum*. Fractions A-4 and A-5 also had a higher MIC at 25°C than at 37°C for *F. necrophorum* (Table 1). Fractions B-2, B-3, B-4 and B-5 had higher MICs at 37°C than at 25°C as did fractions C-5, D-3 and D-4 (Table 1). Control 1% acetic acid did not affect the growth of the organisms at either temperature.

**Figure 1** Effect of chitosan on growth of *Dichelobacter nodusus*. Growth medium was either unsupplemented (■) or supplemented with chitosan A-1 (Extract A with 1% chitosan) at concentrations of 625 mg/L (▲), 1,250 mg/L (●) or streptomycin at a concentration of 128 mg/mL (✱). Bacterial growth was measured at 30 minutes, 1, 2, 6, 24 and 48 hours. Results are expressed by the amount of bacterial growth recovered from 10 µL aliquots of broth subcultured onto agar plates. Agar plates were ranked as: 0 = No growth, 1 = Light growth, 2 = Moderate growth, 3 = Heavy growth.




### *Bacterial survival studies*

After 24 hours of incubation with chitosan or streptomycin sulphate survival of *D. nodusus* was negligible (Fig. 1). Subsequent transmission electron microscopy studies (M McConnell, Unpublished data) have demonstrated that most of the bacteria have ruptured by the 24 hour time period.

## Discussion

Chitosan is a known antimicrobial agent against a wide range of organisms (Vinsova & Vavrikova, 2008). This is the first report of the antimicrobial action of chitosan against the anaerobic organisms implicated in the pathogenesis of footrot. The best overall anti-microbial efficacy at the lowest concentration of a chitosan solution over both temperatures for both organisms was achieved by the A-1, A-2, A-3, and B-2 formulations. Significantly, these results were the equivalent of, or better, than the streptomycin sulphate antibiotic control, in a direct weight per volume comparison.

Molecular weights of chitosan have been reported as being related to the antibacterial activity of chitosan. In general, low molecular weight chitosans appear to be more bactericidal than high molecular weight chitosans but activity does depend on the organism tested (No et al. 2002). In this study the chitosan derivatives used (Molecular weight <211 kDa) were all derived from a chitosan of molecular weight 890 kDa. Previous studies with the Gram negative bacteria *Escherichia coli* and *Pseudomonas fluorescens* have shown chitosan of molecular weight 746k Da to be effective (No et al. 2002). *D. nodusus* is also a Gram negative bacterium and the chitosan used in this study is derived from a similar molecular weight chitosan. Transmission electron microscopy studies with *D. nodusus* have demonstrated that chitosan appears to damage the cell membrane causing peripheral cytoplasm leakage. Damage can be seen within two to four hours incubation of the chitosan with the organism (McConnell, Unpublished data).

An *in vivo* trial in sheep is currently underway to determine if the derivatives are effective under field conditions.

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## References

Ghosh A, Ali MA 2012. Studies on the physiochemical characterisitcs of chitosan derivatives with dicaroboxylic acids. Journal of Material Science 47: 1196–1204.

Hickford JGH, Davies S, Zhou H, Gudex BW 2005. A survey of the control and financial impact of footrot in the New Zealand Merino industry. Proceedings of the New Zealand Society of Animal Production 65: 117–123.

Kennan RM, Han X, Porter CJ, Rood JI 2011. The pathogenesis of ovine footrot. Veterinary Microbiology 153: 59–66.

No HK, Park NY, Lee SH, Meyers SP 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. International Journal of Food Microbiology 74: 65–72.

Tayel AA, Moussa S, Opwis K, Knittel D, Schollmeyer E, Nickisch-Hartfiel A 2010. Inhibition of microbial pathogens by fungal chitosan. International Journal of Biological Macromolecules 47(1): 10–14.

Vinsova J, Vavrikova E 2008. Recent advances in drugs and prodrugs design of chitosan. Current Pharmaceutical Design 14: 1311–1326.