MIC Evaluation of Chlortetracycline, Lasalocid, and Monensin Against *Clostridium perfringens* Isolates of Bovine Origin

Introduction

The genus *Clostridium* is comprised of large gram-positive bacteria, most of which are strict anaerobes. More than 100 clostridial species are identified, with fewer than 20 being pathogenic. They can be grouped into 4 categories, 3 of which are based on type of toxin produced (neurotoxic, histotoxic, and enteropathogenic) and the fourth consisting of clostridia of lesser pathogenic importance.[1] They produce resistant spores and, thus, can be a natural habitat of soil and the intestinal tract of some animals.

When clostridial enteric disease occurs, it is most likely caused by *Clostridium perfringens*. There are 5 types of *C. perfringens* that cause a variety of lesions in various organs. As a group, these syndromes are known as enterotoxemia; an acute, non-infectious, non-contagious disease that originates in the gut of affected animals. Under certain conditions (changing from high-roughage diets to concentrates, drastic increases in milk or concentrate consumption), increased growth of *C. perfringens* occurs, toxins are produced, and enterotoxemia results.

Clostridia produce more toxins than any other bacterial genus, and these toxins are used in identifying them. *Clostridium perfringens* is divided into 5 types based on the types of toxin produced: A, B, C, D, and E. Some *C. perfringens* produce more than one type, but all 5 types produce some toxins in common.[2] It is the exotoxins (found outside the cell wall or in the growth medium) and enterotoxins (those affecting cells of the intestinal mucosa) that account for the local and systemic signs observed with *C. perfringens* infections.[1]

Type A strains are those that produce alpha toxin (a characteristic of almost all isolates) and do not produce other toxins. They are widespread in the intestine of warm-blooded animals and in the environment. Strains of Type A are associated with infections in multiple organs in many species of animals. Lesions in the gastrointestinal tract of cattle infected with Type A *C. perfringens* are well documented. An examination of more than 2500 isolates found Type A to the most predominate type in cattle associated with enterotoxemia.[3] Tympany, abomasitis, abomasal hemorrhage, and ulceration have been associated with Type A *C. perfringens* infections. Diarrhea may or may not occur. Prevalence may approach 40% in certain areas of the US. Sudden death is possible. Some calves may experience diarrhea, clear up for a couple of weeks, and develop the abdominal syndrome 1 to 2 weeks later.[2]

Type A may play a more significant role than previously thought in enteritis of beef calves. The role of *C. perfringens* in the feedlot is less defined, with much of the attention directed towards the possible role of *C. perfringens* Type A in sudden death syndrome.[3] A retrospective study involving 22 dairy cows with hemorrhagic bowel syndrome found that *C. perfringens* was a consistent isolate. Four of the 5 cows from which *C. perfringens* Type A was isolated died.[4]

Type B enterotoxemia is not observed in North America. Type C enterotoxemia, the most common cause of hemorrhagic enterotoxemia in North America, causes acute hemorrhagic enteritis in adult sheep, lambs, calves, kids, and pigs less than 2 weeks of age. Type D enterotoxemia, commonly referred to as “overeating disease,” is the most common cause of enterotoxemia in adult sheep and goats. It is also seen in feedlot cattle, but less frequently than sheep. Affected animals are usually well nourished and on a high plane of nutrition. Type E enterotoxemia, while reported in lambs and calves, is rare.[2]

Clostridium perfringens diagnosis should be considered whenever sudden death occurs in apparently healthy lambs and calves on full feed. In feedlot cattle, the most impressive lesion is a reddish-purple section of small intestine that may be filled with blood. Laboratory confirmation is needed for definitive diagnosis. Presumptive diagnosis can be made when fresh-stained smears of intestinal contents reveal large numbers of gram-positive rods. It must be remembered that *C. perfringens* can be isolated from the intestinal tract of apparently healthy animals.[2]

Study Objective

Studies on minimum inhibitory concentrations (MICs) are used to determine the microbiological activity of compounds against specific pathogens. These studies are useful but it must be understood that in vitro results only aid in the prediction of activity in vivo. Susceptibility testing of anaerobes can be used to determine patterns of susceptibility of anaerobes to new or previously untested antibacterial agents. One must be cautious in setting categories of sensitive, intermediate, and resistant until extensive testing has been performed as directed by the National Committee of Clinical Laboratory Standards (NCCLS).

The objective of this study was the determination of the MIC of selected compounds on *Clostridium perfringens* isolates.

Materials and Methods

Compounds

The following compounds were included in susceptibility testing against a variety of *C. perfringens* isolates of bovine origin: chlortetracycline (CTC), lasalocid (Avatec®/Bovatec®), monensin (Coban®), and combinations of CTC with lasalocid.

Quality control assay values were used to calculate the potencies of the stock solutions of CTC. Pure compounds were used for CTC (Sigma), lasalocid (Alpharma), and monensin (Sigma).

Media

One lot of Difco Wilkins-Chalgren agar dehydrated media (Becton Dickinson; Sparks, MD) was used for agar plates and one lot of Difco Bacto Schaedler broth dehydrated medium (Becton Dickinson) was used for inoculum growth, dilution of inoculum, and as a growth medium for freezing strains at -70°C for long-term preservation. Difco tryptic soy agar dehydrated medium (Becton Dickinson) supplemented with 5% defibrinated sheep blood (Remel; Lenexa, KS) was used for routine subculturing of isolates.

Strains

Animal-derived strains were obtained from several sources. The University of Arizona Department of Veterinary Science and Microbiology (Tucson, AZ) provided 10 bovine isolates (calf). South Dakota State University (Brookings, SD) provided 39 bovine isolates (mostly dairy cattle) and 1 equine isolate. Texas Veterinary Medical Diagnostic Laboratories (Amarillo, TX) provided 17 bovine isolates. In total, 66 isolates were used in this study. The UAZ isolates were all type A. Of the SDSU isolates, 38/39 were type A with 3/38 carrying the beta-2 toxin band; 1/39 was type C. The TVMD Laboratories reported 11/17 isolates were type A with 5/11 carrying the beta-2 toxin band. Six of the TVMD Laboratory isolates were not tested for genotype (undetermined types). Thus 59/66 isolates were type A and 8/59 type A beta 2, 1/66 was type C, and 6/66 of undetermined genotype.

All *C. perfringens* strains were recovered from frozen condition. Four American Type Culture Collection *C. perfringens* strains (ATCC 13124, 3828, 43402, and 25768) were selected to act as control strains, and in addition NCCLS suggested quality control strains of *Bacteroides fragilis* (ATCC 25285), *Bacteroides thetaiotaomicron* (ATCC 29741), and *Eubacterium lentum* (ATCC 43055) were tested.

Inoculum preparation

All isolate and quality control strains were recovered from frozen condition and subcultured twice on tryptic soy agar supplemented with 5% defibrinated sheep blood. After the second subculture, single colonies were picked and inoculated into tubes of Schaedler broth supplemented with Oxyrase™ (Oxyrase, Inc.; Mansfield, OH) to generate anaerobiosis. Inoculum was grown at 37°C for 6 to12 hours then diluted in Schaedler broth to a 0.5 McFarland turbidity standard equivalence and used within 0.5 hours of dilution. All other incubations were at 37°C for 24 hours using BBL Gas Pak™ jar systems (BD Diagnostic Systems; Sparks, MD) to generate anaerobic conditions.

TABLE 1. Minimum inhibitory concentrations (µg/mL) against NCCLS quality-control strains and 4 *Clostridium perfringens* (CP) ATTC strains.*

Strain	CTC	MON	LAS	CTC/LAS
BT	2.0	2.0	1.0	0.13
BF	0.06	2.0	1.0	0.13
EL	2.0	2.0	0.13	0.13
CP25768	<.015	2.0	0.13	0.13
CP13124	0.03	2.0	0.13	0.13
CP3628	0.03	2.0	0.13	0.13
CP43402	<.015	2.0	0.13	0.13

*agar dilution susceptibility testing
BT=*Bacteroides thetaiotaomicron*, BF=*Bacteroides fragilis,* EL=*Eubacterium lentum*

Antimicrobial stock solutions

Chlortetracycline and monensin were obtained from Sigma (St. Louis, MO). Lasalocid was obtained from Alpharma (Willow Island, WV). The potencies were determined using label claims for potency. Stock solution diluents were not specified in the NCCLS guidelines for any of these compounds. Chlortetracycline was dissolved in 25 mL 0.1*N* HCl and brought up to volume with pH 4.5 phosphate buffer. Lasalocid and monensin compounds were dissolved and brought up to final volume of 50 mL in dimethyl sulfoxide (DMSO). Chlortetracycline was filtered through a 0.45-µm low-protein binding sterile acrodisc syringe filter (Gelman Sciences; Ann Arbor, MI) for sterility. A stock solution was freshly prepared and dilutions were prepared using appropriate phosphate buffer for CTC. Lasalocid and monensin dilutions were prepared using sterile deionized water because of solubility issues.

Agar dilution assay for antimicrobial activity

The NCCLS M11-A4 schemes for preparing dilutions of antimicrobial agents to be used in agar dilution susceptibility tests were followed. Media were prepared according to manufacturer guidelines. Control plates were utilized as described by NCCLS document M11-A4. In addition, agar plates were prepared which incorporated 2 mL DMSO into 18 mL agar and inoculated after control plates. The DMSO-containing plates were designed to test for possible inhibitory effects on the growth of the *C. perfringens*.

Results

The quality control and field isolate results are summarized in Tables 1 and 2, respectively. Not all isolates were tested against CTC (65/66). None of the organisms tested appeared to be sensitive to DMSO when diluted to the NCCLS-recommended concentration for susceptibility testing. The MIC_{90} of the compounds against *C. perfringens* field isolates were as follows: CTC, 8 µg/mL; monensin, 2 µg/mL; lasalocid, 0.13 µg/mL.

Conclusions

Aureomycin® (CTC) expressed the lowest MIC of all products tested, with 3 isolates revealing a MIC of < 0.03 µg/mL, and 8 isolates with MICs of 0.03 µg/mL.

Lasalocid appeared to be slightly more effective than monensin in this study. Based on these results, lasalocid may be more effective than monensin in controlling *C. perfringens*-associated disease. Field studies, however,

TABLE 2. Minimum inhibitory concentrations (MIC) of various *Clostridium perfringens* from bovine origins.*

	Number of strains per MIC (µg/mL)												
Compound tested	< 0.03	0.03	0.06	0.13	0.25	0.50	1	2	4	8	16	32	>32
Chlortetracycline	3	8	2	–	1	4	7	8	15	17	1	1	1
Monensin	–	–	–	–	–	–	–	66	–	–	–	–	–
Lasalocid	–	–	–	66	–	–	–	–	–	–	–	–	–
CTC 0.03/Lasalocid	–	–	–	66	–	–	–	–	–	–	–	–	–

*agar dilution susceptibility testing
MIC range of the *Clostridium perfringens* ATCC strains used as controls is shaded for each compound

are needed to determine the efficacy of lasalocid in prevention or control of the disease.

The combination of sub-MIC levels of CTC with lasalocid at a full range of concentrations indicated that the antibacterial effect was additive and not competitive or synergistic. Further in vitro studies are needed to confirm lasalocid and CTC MIC values for *C. perfringens*.

It has been previously demonstrated that lasalocid, when compared to monensin, exhibits improved feed intake,[5,6] coccidiosis efficacy, and product safety.[7] This study suggests additional benefits may be realized when utilizing lasalocid.

References

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