In vitro testing of antimicrobial agents for proliferative enteropathy (ileitis)

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Summary: *Proliferative enteropathy (ileitis) is a common disease of grower and finisher pigs. Recent studies by the authors have illustrated reliable methods of in vitro culture of the etiologic agent, an obligate intracellular bacterium, known as ileal symbiont (IS) intracellularis. Characteristic lesions of proliferative enteropathy are reproduced when IS intracellularis is used as oral inocula in challenge experiments in pigs. No clear pattern of the antibiotic sensitivity of IS intracellularis has emerged during clinical usage of antibiotics in field treatment situations over the past 20 years, and few controlled treatment trials have been reported. We used the in vitro cell culture system necessary for the growth of IS intracellularis to test the in vitro effect of 18 antimicrobial agents. The minimum inhibitory concentration (MIC) of each agent was determined for up to three strains of IS intracellularis. The minimum bactericidal concentrations (MBC) of selected agents were determined for one strain. Penicillin, erythromycin, difloxacin, virginiamycin, and chlortetracycline were the most active compounds tested, each with an MIC of < 1 µg per mL. Tiamulin and tilmicosin were the next most active compounds, with MICs of < 4 µg per L. These results indicate that compounds capable of entering the host cell cytoplasm and blocking protein synthesis, such as macrolides, tetracyclines, fluoroquinolones, and virginiamycin are active against IS intracellularis in vitro. Some of these drugs have previously been recommended clinically for the treatment of ileitis. The MICs of aminoglycosides, aminocyclitols, ceftiofur, bacitracin, and avoparcin were generally greater than their normal recommended dose rate, usually >32 µg per mL. The MBC results were broadly similar to the MIC results for the aminoglycosides and other groups of agents tested. However, the aminoglycosides may have some effect in controlling secondary infections to a primary IS intracellularis-induced lesion of the intestine. There is clear need for in vivo treatment trials in experimental and field situations to support a list of drugs that is likely to be effective in the treatment and control of ileitis.*

Proliferative enteropathy (ileitis) is a common disease of grower and finisher pigs. It occurs under a variety of management systems, and is particularly noticeable in herds of high health status. Clinical manifestations usually include poor growth rates and runting in growing pigs and bloody scours and/or sudden death in finisher pigs.[1] Characteristic lesions at necropsy of affected pigs are gross hyperplasia of the mucosa of the ileum and/or colon, with or without associated hemorrhage. Histologically, a consistent finding within these lesions is the presence of numerous intracellular bacteria, currently known as ileal symbiont (IS) intracellularis, located free in the enterocyte cytoplasm.[2–4] Reliable methods of in vitro culture of IS intracellularis, using an enterocyte cell culture system, have recently been developed.[5] Clear lesions of proliferative enteropathy are reproduced by orally inoculating pigs with cultured IS intracellularis.[6,7] The similar reactions of IS intracellularis-specific monoclonal antibodies and DNA probes with IS intracellularis strains from North America, Europe, and Australia have confirmed the identity of IS intracellularis in proliferative enteropathy lesions worldwide.

In the 20 years since the consistent presence of the intracellular bacteria in this disease was first identified, no clear pattern of its antibiotic sensitivity has emerged. Few controlled treatment trials have been reported. In one, Peter Beers and Bob Love demonstrated that 52 of 145 untreated control pigs were affected with proliferative enteropathy during a severe outbreak, but that none of 144 similar, exposed pigs treated with 100 ppm oxytetracycline were affected.[8] In another trial, again during a severe outbreak, Melissa Fleck Veenhuizen demonstrated a clinical response in pigs treated with 100 ppm tylosin, but not in untreated pigs, or those given 40 ppm tylosin.[9] Several other reviews and reports have provided speculative clinical impressions of drugs that may be beneficial to pigs affected with proliferative enteropathy, but without incorporating any controls.[10–12] These reports also gave clinical recommendations for treating ileitis in pigs. The development of in vitro culture methods for IS intracellularis now allows the determination of minimum inhibitory concentration (MIC)

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and minimum bactericidal concentration (MBC) data, which may then be related to in vivo trials and field data. This would allow the development of more definitive treatment and control regimes.

While standard Kirby-Bauer disk diffusion tests are not applicable to obligate intracellular bacteria such as IS intracellularis, the use of the in vitro cell culture method may provide a less remote guide to the effects of antibacterial agents, as it more closely mimics any reaction within the body. Due to the tedious nature of the test system, few strains were available for full testing.

Materials and methods

Antimicrobial agents

Antimicrobial agents purchased from Sigma Chemical Co. (Poole, United Kingdom) were neomycin, gentamicin, penicillin G procaine, ampicillin, spectinomycin, avoparcin, and bacitracin-zinc. Antibiotics kindly supplied by the manufacturers were tylosin, tilmicosin, and apramycin (Elanco Animal Health, Indianapolis, Indiana), ceftiofur and lincomycin (Upjohn Co., Kalamazoo, Michigan), erythromycin and difloxacin (Abbott Laboratories, North Chicago, Illinois), virginiamycin m/s (Pfizer, New York City, New York), enrofloxacin (Bayer plc, Bury St. Edmonds, United Kingdom), chlortetracycline (American Cyanamid, Wayne, New Jersey) and tiamulin (Sandoz-Biochemie, Kundl, Austria). Antibiotic stock solutions were prepared immediately prior to use. Virginiamycin m/s, tilmicosin, and erythromycin were dissolved first in one mL of ethanol, then diluted with distilled water; the amount of ethanol introduced into cultures (<10 ppm) did not inhibit growth. Enrofloxacin and difloxacin were dissolved first in one mL of alkaline water (pH 10), then diluted in distilled water. All stock solutions were sterilized by filtration and then diluted to provide the desired concentration when added in a 0.5 mL of cell culture.

Table 1

MICs for antimicrobial agents for IS intracellularis in the in vitro cell culture system

Antibiotic	No. of strains tested	Intracellular activity assay MIC (μg/mL)	Extracellular activity assay MIC (μg/mL)
Peptides			
Bacitracin-zinc	1	> 32	> 32
Avoparcin	1	> 64	> 64
Virginiamycin m/s	2	1	1
Penicillins			
Penicillin G procaine	2	1	2
Ampicillin	2	1	8
Tetracycline			
Chlortetracycline HCl	2	1	16
Pleuromulin			
Tiamulin hydrogen fumarate	3	4	4
Macrolides			
Erythromycin thioCN	2	0.1	0.5
Tylosin tartrate	3	64	64
Tilmicosin	2	2	2
Lincosamide			
Lincomycin HCl	2	32	32
Aminocylitols			
Apramycin sulfate	1	> 128	128
Spectinomycin diHCl	1	32	32
Aminoglycosides			
Neomycin sulfate	3	> 128	128
Gentamicin sulfate	2	> 128	128
Cephalosporin			
Ceftiofur sodium	1	> 8	> 8
Fluoroquinolones			
Enrofloxacin	2	8	8
Difloxacin HCl	2	0.1	0.5

IS intracellularis culture

Three strains of IS intracellularis isolated from lesions of proliferative enteropathy were cultured in IEC-18 rat enterocyte cultures as detailed elsewhere.[5] For antimicrobial sensitivity testing, small vials containing monolayers of IEC-18 cells were infected with approximately 10^4 IS intracellularis in 0.5 mL inoculum and incubated microaerobically (8% oxygen, 8.8% carbon dioxide) for 5 days at 37°C. Each test antimicrobial agent was added to triplicate cultures for each concentration and strain tested.

To test the inhibition of intracellular IS intracellularis growing within enterocytes (a 'treatment' strategy), antibiotic was only added to medium used for replacement, 1, 2, and 3 days after commencement of infection. To test the inhibition of extracellular IS intracellularis prior to and during the infection process (a 'prevention' strategy), antibiotic was only added to medium used at the time of infection, which contained the IS intracellularis inoculum. When this medium was replaced 1, 2, and 3 days later, no antibiotic was added. In separate control cultures, no antibiotic was added at any stage.

To assess the number of IS intracellularis present after 5 days incubation, each cell culture was stained in an indirect immunoperoxidase assay, incorporating a specific monoclonal antibody. The total number of heavily infected

cells (>30 bacteria per cell) was counted for each culture. Stained IS intracellularis growing in cells are illustrated in reference 5. A data matrix comprising the bacterial counts for each culture was set up. To facilitate comparisons between the numerous datapoints, results were expressed as a percentage ratio of the bacterial count for each culture tested for intracellular inhibition or for extracellular inhibition of IS intracellularis, divided by the mean count for the relevant control cultures. Therefore antibiotics totally inhibiting growth would have a ratio value of 0%, while those not affecting growth would have a value of nearly 100%. An antibiotic's MIC was then taken as the endpoint concentration where the IS intracellularis growth fell below 1% of the control cultures.

Minimum bactericidal concentration

One-day monolayers of dividing IEC-18 were prepared as described above. Batches of one IS intracellularis strain were used as inocula. Inocula were initially added to cells in culture medium free of antibiotics and incubated for 1 day as described above. On day 1, all vials were removed from the incubator, and infected cells re-fed with fresh medium, either containing test antibiotic or not, and replaced in the incubator. On days 2 and 5, all vials, test and controls, were removed and re-fed fresh medium without antibiotics in any vial. On day 7, all vials were removed and cells on coverslips assessed for IS intracellularis infection as described above. The MBC would be the lowest concentration where use of a 'pulse' treatment of antimicrobial agent stopped the growth of IS intracellularis.

Results and discussion

The MICs of 18 antimicrobial agents for IS intracellularis in the in vitro cell culture system are given in Table 1, and the MBCs for nine agents are given in Table 2. While only a small number of strains were tested, limiting the test's ability to fully predict in vivo therapeutic outcomes, some likely conclusions were indicated.

For several agents, the MIC/MBC determined was greater than a likely dose rate for use of the drug in pigs. Thus it is unlikely that bacitracin, avoparcin, ceftiofur, or any aminoglycoside or aminocyclitol antibiotic could achieve in vivo levels comparable with their MIC/MBC. The known pharmacological actions of these drugs make this finding unsurprising, as bacitracin and avoparcin are generally only capable of inhibiting the growth of Gram-positive bacteria, and aminoglycosides and aminocyclitols are generally only capable of inhibiting the growth of aerobic organisms.[13] In contrast, IS intracellularis is a microaerobic, Gram-negative organism, which grows best at 8% oxygen.[5] The normal oxygen tension in the porcine ileum is 5%–10% oxygen.[14] Also, aminoglycosides tend to locate specifically in cell lysosomes,[15] whereas IS intracellularis is located in the cell cytosol compartment.[1,2] A few clinical reports have suggested that bacitracin, or aminoglycosides such as neomycin, are useful in the treatment of ileitis, with brief mention of possible dose rates.[10–12] Our MIC/MBC results would not support the clinical use of these drugs for direct action against IS intracellularis. If an effect of these drugs on ileitis were to be envisaged, then it is likely to be due to an effect on secondary infections in the affected bowel. These are considered relatively common in ileitis, often manifesting as a necrotic enteritis.[1] Secondary infections in ileitis may occur due to the mucosal thickening caused by the primary etiologic agent IS intracellularis, allowing gut stasis and overgrowth by organisms normally present in low numbers, such as *Campylobacter* spp.

Table 2

MBCs for selected antimicrobial agents for IS intracellularis strain NCTC 12656 in the in vitro cell culture system

Antibiotic	MBC (µg/mL)
Peptide	
Virginiamycin m/s	< 2
Penicillin	
Penicillin G procaine	4
Tetracycline	
Chlortetracycline HCl	4
Pleuromulin	
Tiamulin hydrogen fumarate	< 2
Macrolide	
Tylosin tartrate	4
Aminoglycosides	
Gentamicin sulfate	> 50
Fluoroquinolones	
Enrofloxacin	4
Difloxacin HCl	< 2

Other test antibiotics, such as penicillin and fluoroquinolones, were found to have low MIC and MBC values, but have not been widely recommended to treat ileitis. This may merely reflect a fashionable preference for other drugs in swine practice, due to factors such as availability, and/or their lack of field evaluation in swine medicine. Alternatively, there could be true problems with these drugs due to inappropriate pharmacodynamics of each drug, i.e., the drug may not penetrate to the site of the organism in the bowel after a full dosing regime. However, our in vitro data do concur with previous studies indicating that penicillins and fluoroquinolones can have good activity against bacteria located within the cell cytoplasm.[15,16] Only in vivo evaluation of these drugs in animals challenged with IS intracellularis in natural and experimental situations will clarify the situation for each drug. While pigs are the main host species for ileitis, a nearly identical disease occurs in the laboratory hamster, and these animals may serve as a useful model for infection. Cross-species transmission of the lesions occurs if pig-derived IS intracellularis are given to hamsters.[17] However, any results obtained in hamsters would need to be confirmed in pig trials.

Lastly, some test antibiotics such as macrolides, virginiamycin, tetracyclines, and tiamulin, which have been previously recommended for the treatment of ileitis,[10–12] were found to have low to

moderate MIC/MBC values. These groups of antibiotics act by inhibiting protein synthesis of bacteria, a method of action not dependent on aerobiasis. The few controlled treatment trials conducted for ileitis have demonstrated the probable effectiveness of tetracyclines and macrolides at appropriate doses in some situations.[8,9] Both of these groups of antibiotics are also known to be effective in treating other intracellular bacterial infections in vivo,[16,18] reflecting other in vitro studies which have demonstrated that these drugs can become concentrated in the cell cytoplasm.[15,16,19] The apparent variation between some macrolides in the intracellular activity against IS intracellularis has also been noted in in vitro MIC studies of other intracellular bacteria.[15] The likely clinical effect of macrolides and tetracyclines for ileitis, as suggested by controlled treatment trials, has now been supported by the in vitro cell culture MIC/MBC determinations. It is therefore likely that our MIC/MBC results broadly reflect the true susceptibility of IS intracellularis to the other drugs tested. The inherent difficulties in relating an in vitro test, albeit a close model of the in vivo situation, to the clinical use of each drug does mean that results should be interpreted with some caution.[20] We therefore look forward to a day when an effective treatment and control program for ileitis can be confidently recommended, based on the correct antibiotic usage and management practices.

Implications

- The primary etiology of proliferative enteropathy (ileitis) is IS intracellularis, an obligate intracellular bacterium.
- IS intracellularis is susceptible to macrolides, tetracyclines, and some other drugs in vitro, but not aminoglycosides.
- In vivo trials will determine the optimum treatment regimes.

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