Isolation, identification, and antimicrobial susceptibility of *Clostridium perfringens* isolates from acute necrotic enteritis of broiler chickens

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Abstract

The aim of this study was to isolate, identify and determine the antimicrobial susceptibility of *Clostridium perfringens* (CP) isolates from acute necrotic enteritis of broiler chickens. All broiler carcasses diagnosed as necrotic enteritis (NE) were sampled, subjected to microbial tests and 40 isolates were identified according to standard procedures. The antimicrobial susceptibility of CP isolates to 20 antibacterial agents was then determined. The results show widespread resistance among CP isolates. The most frequent resistance was observed to neomycin sulfate (87.5%), and then to lincomycin and tetracycline (both 80%). No isolate was resistant to chloramphenicol and the least frequency of resistance was observed to vancomycin (10%), sulfamethoxazole+trimethoprim (17.5%), and penicillin (20%). All isolates were multiple drug resistant types. There were 39 resistant patterns among the CP isolates, 95% of which were distributed in 38 resistant patterns. These multiple and variable resistance patterns observed among the CP isolates, even among different isolates from one farm, demonstrate a challenge for veterinarians in the field to choose the correct compound to combat the occurrence of NE.

Introduction

Clinical necrotic enteritis (NE) is one of the bacterial diseases, found primarily in young chickens, produced by *Clostridium perfringens* (CP) type A and, to a lesser extent, type C (Prescott *et al*., 1978; Shane *et al*., 1985; Anett *et al*., 2002; Van Immersal *et al.*, 2004; Opengart, 2008). Both CP types are known to produce toxins: type A, alpha toxin and type C, both alpha and beta toxins (Shane *et al.*, 1985; Van Immersal *et al.*, 2004). Since in-feed antibiotics and ionophores are effective in the prevention and treatment of the disease, after the ban on the use of growth promoter antibiotics and ionophore anticoccidials in the European union (EU), NE has become one of the most important threats to the broiler industry in the EU (Casewell *et al.*, 2003; Grave *et al.*, 2004; Chalmers *et al.*, 2007). In the US, when broiler producers reduced the usage of growth promoter antibiotics, different Clostridial diseases began to increase (Shane, 2004). However, in some countries, where growth promoter antibiotics and ionophores are still utilized for poultry, the occurrence of NE is not as common as in EU countries, which have banned the use of these drugs. It still remains a challenge for countries in which the ban is in place to find an effective antibacterial agent to combat this deadly disease. A number of studies have shown the role of antibiotic-supplemented feeds on the development of resistant strains to antibacterial agents (Rood *et al.*, 1978; Summanen *et al.*, 1993). This resistance may develop because the use of antibiotics in feeds has led to the selection of resistant bacteria (Rood *et al*., 1978).

In spite of having knowledge about many predisposing factors to NE (Williams, 2005), when facing the disease, veterinarians have to administer an appropriate antibiotic to birds to reduce the mortality rate, as well as other detrimental effects of the disease. Therefore, determining the antimicrobial susceptibility of CP isolates from NE outbreaks is very important. In this study, 40 CP isolates recovered from acute clinical NE cases were characterized for their antimicrobial susceptibility patterns.

Materials and Methods

Isolation and identification of *Clostridium perfringens* (CP)

The carcasses of all broiler chickens diagnosed as necrotic enteritis (NE) (the presence of typical

fibrinonecrotic lesions in the mucosal membrane of the intestines) were sampled and subjected to microbial tests. The intestinal serosal surface was sterilized with a hot spatula. An incision was then made and a part of the mucosal surface of the intestine was taken by a sterile loop for a smear and gram stain. Identification of the bacteria was performed according to procedures described by Summanen *et al*. (1993), Quinn (1994), Miller (1998). A presumptive diagnosis of CP was made for Gram-positive, spore-containing bacteria. These samples then were streaked onto blood agar (BA) plates and placed in anaerobic jars (Merck, Germany) containing commercial gas pack (Anaerocult A, Merck). The jars were closed and incubated at 37°C for 48 h. The indicator strips (Anaero-test, Merck) were included in each jar to confirm the anaerobic conditions. After 48 h, the BA plates were examined for colony morphology. Observation of large, smooth and round colonies with 2-4 mm in diameter having double hemolysis (complete hemolysis in the inner zone and incomplete hemolysis in the outer zone) were considered as a presumptive diagnosis of CP. The colonies were then checked by Gram-staining of the colonies was observed under the microscope. The suspected positive samples were screened for lecithinase, lipase, urease and indole production, motility, and reverse-CAMP test. Finally, the suspected colonies were cultured onto Triptone Sulfite Neomycin (TSN; Merck) agar plates. TSN-inoculated plates were incubated anaerobically at 37°C for 18 h. Dark-centered colonies were considered as containing CP.

Antimicrobial susceptibility test

The susceptibility of 40 CP isolates to a panel of antimicrobial agents was determined as previously described (Quinn *et al*., 1994). The antimicrobial agents that were tested, and their concentrations (µg) were as follows: difloxacin (10), ofloxacin (5), norfloxacin (10), enrofloxacin (5), nalidixic acid (30), flumequine (30), penicillin (10), ampicillin (10), amoxi-clav (30), neomycin (30), gentamicin (10), lincomycin (30), lincospectin (15/200), erythromycin (10), tylosin (30), chloramphenicol (30), tetracycline (30), colistin (10), vancomycin (30) and trimethoprim-sulfamethoxazole (1.25/23.75). In this study, the CP isolates with intermediate susceptibility classification were considered not to be resistant to that drug and the multi-resistance was defined as resistance to more than one drug.

Results

In the present study, the resistance to antibacterial compounds was found to be widespread among the CP isolates. The most frequent resistance was observed to neomycin sulfate (87.5%), and then to lincomycin and tetracycline (both 80%; Table 1). No isolate was resistant to chloramphenicol and the least frequency of resistance was observed to vancomycin (10%), sulfamethoxazole+trimethoprim (17.5%) and penicillin (20%; Table 1). All isolates were resistant to more than one antibacterial agent. More than 50% of isolates were resistant to more than five drugs and one isolate (2.5%) showed multiple resistances to more than 14 drugs. There were 39 resistant patterns observed to 20 tested antibacterials among the CP isolates that were tested. Thirty-eight (95%) isolates each showed an individual resistance patterns. Only two isolates (5%) showed an identical pattern of resistance.

Discussion

Different antibacterials have been used for the treatment, or as in-feed growth promoters for the prevention, of NE outbreak in poultry (Prescott *et al*., 1978; Hamdy *et al*., 1983). The susceptibility of CP isolates to different sources of antibacterials has been studied by many and variable results have been obtained.

Jung *et al*. (1983) evaluated the sensitivity of 50 CP isolates from human feces to cephotaxim, fosfomycin, penicillin-G and vancomycin. They observed no resistance to pen-G or cephotaxim, but did observe variable resistance to other agents. Devriese *et al*. (1993) studied the minimum inhibitory concentration of seven growth promoter antibacterials against 95 CP isolates from poultry, pigs and calves. These researchers found resistance to bambermycin and flavomycin (flavophosfolypol) and susceptibility to avoparcin, avilamycin, and salinomycin among all 95 isolates. Resistance to tylosin and virginiamycin

Table 1: Antimicrobial susceptibility test results of 40 *Clostridium perfringens* isolates from cases of necrotic enteritis.[a]

	Antimicrobial drugs	S	I	R
1	Vancomycin (Vc)	90	0	10
2	Erythromycine (Er)	2.5	67.5	30
3	Tylosin (Ty)	25	47.5	27.5
4	Amoxi-Clav (Amx)	70	0	30
5	Ampicllin (Amp)	40	32.5	27.5
6	Penicillin (Pen)	80	0	20
7	Gentamicin (Gen)	47.5	0	52.5
8	Flumequine (Flu)	52.5	7.5	40
9	Colistin (Col)	12.5	47.5	40
10	Tetracycline (Tet)	7.5	12.5	80
11	Chloramphenicol (Chl)	82.5	17.5	0
12	Lincomicin (Lin)	20	0	80
13	Linco-spectin (LP)	57.5	10	32.5
14	Ofloxacin (Ofx)	50	10	40
15	Norfloxacin (Nor)	67.5	10	22.5
16	Enrofloxacin (Nfx)	37.5	30	32.5
17	Neomycin (Neo)	5	7.5	87.5
18	Nalidixic acid (NA)	35	12.5	52.5
19	Difloxacin (Dfx)	70	2.5	27.5
20	Trimethoprim- Sulfamethoxazole (SXT)	82.5	0	17.5

[a]S = Susceptible, I = Intermediate Susceptible, R = Resistant

among isolates from different sources, and resistance to bacitracin in some of poultry and calf isolates, was also observed. Cummings *et al.* (1995) conducted a farm survey and found resistance to lincomycin and bacitracin and sensitivity to penicillin. Sasaki *et al.* (2001) isolated some *Clostridium* species from diseased cattle and reported a 71% resistance to tetracycline in the CP isolates. Martel *et al.* (2004) studied the sensitivity of CP isolates, which had been isolated from 31 different Belgian broiler farms, to 12 antibacterials and reported a high level of resistance to lincomycin and tetracycline. Johansson *et al.* (2004) observed 76%, 29%, and 10% resistance to tetracycline among CP isolates from Sweden, Norway, and Denmark, respectively. The high level of resistance to tetracycline in Sweden is interesting because this antibiotic was rarely used in Swedish broiler farms. Kather *et al.* (2006) studied the prevalence of tetracycline resistant genes in 124 CP isolates from dogs in the United States and found a relatively high prevalence of *in vitro* resistance to tetracycline. The high level of resistance to lincomycin and tetracycline was also observed among the CP isolates in this study. The high level of resistance to lincomycin can be attributed to resistant genes that had not been detected. Transfer of tetracycline resistance has already been documented in *Clostridia* (Tally and Malamy, 1982).

In a survey performed from 1986 to 2002 in northern Europe, 100% of CP isolates were found to be sensitive to vancomycin (Johansson *et al*., 2004). In this study, a 90% sensitivity was observed in the CP isolates to vancomycin. Tansuphasiri *et al*. (2005) examined the antimicrobial susceptibility among 201 CP isolates from the feces of humans and pigs, food, and other environmental sources. These researchers showed resistance to tetracycline (56.2%) followed by imipenem (24.9%), metronidazole (9.5%), penicillin G (9%), vancomycin (4.5%), chloramphenicole (3%) and ceftriaxone (1%) among the isolates. Most of the isolates from pig feces (77.8%), the environment (72.7%), human feces (44.9%) and food (28%) showed resistance to tetracycline. The low level of resistance to vancomycin and penicillin G observed in this study was comparable to findings of Tansuphasiri *et al.* (2005). In a study by Johansson *et al*. (2004), 100% susceptibility to ampicillin was been reported among CP isolates, while a much lower susceptibility was observed to ampicillin.

The reason for sensitivity to some antibiotics can be explained by the level of their usage in poultry farms (Tansuphasiri *et al*., 2005). In this study, a high level of sensitivity to vancomycin and penicillin G was observed. These antibiotics are not used in Iranian poultry farms. Likewise, tetracycline, which is a commonly used antibiotic in Iranian poultry farms, was the drug to which a very high resistance was observed. One major drawback in monitoring of resistance to CP is the anaerobic conditions that are required for bacterial growth. Since culture and antimicrobial susceptibility tests for anaerobic CP are not routinely used in diagnostic laboratories of this country, in case of NE outbreaks blind treatments are performed, which may lead to the inappropriate and incorrect prescription of antibiotics and, therefore, the rise of resistance to CP. The widespread resistance patterns observed among the CP isolates in this study indicates the diverse groups of CP isolates circulating in broiler farms and the possible variability of response in the *in vitro* test method used for these anaerobic bacteria.

Multiple drug resistant (MDR) types are commonly found among CP isolates. Dutta and Devriese (1981) found different drug resistant patterns against macrolide-lincosamide and streptogramin in CP isolates of animal origin. Tansuphasiri *et al*. (2005) studied antimicrobial resistance among *Clostridium perfringens* isolated from the feces of humans and pigs, food and other environmental sources. They reported that among 62.7% of antimicrobial resistant strains, 39.3% were resistant to a single drug and 23.4% were MDR strains; of 47 MDR strains, 63.8% were derived from human feces and were resistant to between two and six drugs. Traub *et al*. (1986) found that three of 106 CP isolates had MDR against clindamycin, erythromycin, josamycin, tetracycline and, in one case, against chloramphenicol. Rood *et al.* (1978) also observed CP isolates that were MDR strains. These isolates were resistant to tetracycline, erythromycin, clindamycin and lincomycin. However, none of the isolates were resistant to penicillin or chloramphenicol. These resistant patterns are very similar to the results obtained in this research. Rood *et al*. (1978) also found that resistance to erythromycin was always associated with resistance to lincomycin and clindamycin. In this study, all the isolates were MDR strains, nine (22.5%) isolates were resistant to more than ten antibacterials, and one (2.5%) isolate showed resistance to 14 antimicrobial agents. It should be noted that resistance patterns are local phenomenon and using antibacterials according to patterns of other regions may be misleading and inappropriate.

The resistance mechanisms of anaerobic bacteria to antibacterials have been studied by some researchers (Finegold, 1989; Rood *et al*., 1978). Rood *et al*. (1978) have shown that plasmids are the cause for resistance of bacteria to many kinds of antibiotics. Since plasmids can be transferred between bacteria of the same, and other, species, and they can carry with them the resistance genes to many antibacterials, resistance may become widespread. Finegold (1989) specified other types of resistance encountered in anaerobic bacteria including the following: the production of beta-lactamase enzymes, inactivating enzymes such as chloramphenicol acetyltransferase, plasmid-mediated transferable MDR, changes in porin molecules in the

outer membrane of the bacterial cell, decreased uptake of drug by other mechanisms, changes of the target organs such as penicillin binding proteins and a reduction of the antibiotic to an active intermediate product.

The multiple and variable resistance patterns observed in this study among the CP isolates, even among different isolates from the same farm, demonstrate the challenge faced by veterinarians in the field in choosing the correct compound to combat NE. The use of automatic or semi-automatic systems to identify the CP isolates, performing antimicrobial susceptibility test and evaluating an appropriate number of field samples could all play a part in determining a more accurate resistance pattern of an affected flock.

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