Haryana Vet. **48** (December, 2009), pp 81-84 Research Article

IN VITRO LECITHINASE ACTIVITY AND ANTIBIOGRAM OF CLOSTRIDIUM PERFRINGENS ISOLATED FROM BROILER CHICKENS

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ABSTRACT

Clostridium perfringens is well known as the etiological agent of necrotic enteritis in chickens. *C. perfringens* were detected in intestinal contents from forty nine cases of enteritis in broiler chicken. In the present study *in-vitro* lecithinase activity of 49 isolates and sensitivity of 30 isolates to 11 antimicrobial agents were assessed. A total of forty four (89.79%) isolates expressed lecithinase activity. Antibiogram studies showed highest sensitivity to fluroquinolones like ciprofloxacin (93.3%), followed by enrofloxacin (86.6%) and moderate sensitivity to chloramphenicol (76.6%), oxytetracycline (70%) and co-trimoxazol (63.3%). The organisms showed 100% resistance to streptomycin and neomycin.

Key words: *Clostridium perfringens*, lecithinase activity, antibiogram

Clostridium perfringens is the normal bacterial flora of gastrointestinal tract in both human and animals. It has also been shown to cause a number of diseases in humans and animals and reported to be a causal agent of necrotic avian enteritis through out the world. The types of *C. perfringens* involved in this disease are type A and type C namely (Parish, 1961, Long, 1973). *C. perfringens* possess lecithinase activity due to phospholipase C of alpha toxin of *C. perfringens*. There are many antibiotics available for medical and veterinary use and some are used as growth promoting agents. For broiler chickens feed additive antibiotics including penicillin, tetracyclin, bacitracin have been used. The *in-vitro* susceptibility of *C. perfringens* to antibiotics has been described by many workers (Traub, 1971 Tally *et al.*, 1975, Dutta and Devriese, 1980). However, most of these reports were limited mainly to commonly available antibiotics and to only a few strains of *C. perfringens* originating from necrotic enteritis in broiler chickens. Moreover there is no report of isolation and antibiogram of *C. perfringens* isolates from broiler chicken in the country. This communication describes the *in-vitro* lecithinase activity and antibiogram assay of *C. perfringens* isolated from enteritis affected cases of broiler chickens.

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MATERIALS AND METHODS

Isolation of *C. perferingens*: Reference strains of *C. perfringens* type 'A' and type 'C' were procured from the Division of Biological Standardization, Indian Veterinary Research Institute (IVRI), Izatnagar. The strains were maintained as stock culture in thioglycollate broth and were periodically tested for purity, morphology and biochemical characteristics. Anaerobic jars were used for anaerobic incubation wherever required. Burning candle was used in anaerobic jar and it was tightly sealed with parafilm to create anaerobic environment. The intestinal contents collected from 4-5 enteritis affected birds in a flock were pooled to make one pooled sample (pooled sample will be referred to as sample in the manuscript). Sixty eight pooled samples of intestinal contents were processed for isolation of *C. perfringens* using one step enrichment and selective plating (Varnam and Evans 1991). Ten gram of faecal sample was inoculated in 90 ml of thioglycollate enrichment medium (Hi media) in sterile flask. After mixing for 2-3 min, homogenized sample was heated at 75°C for 20 min, followed by incubation at 37°C for 24 h. For isolation of *C. perfringens,* an agar overlay technique in sterile test tubes was used using Tryptose Sulphite Cycloserine agar [TSC] (Varnam

and Evans 1991). Thioglycollate enriched inoculum (0.1ml) was taken in a sterile straight test tube and 5 ml of sterile TSC agar was added with thorough mixing. It was allowed to solidify for 5-10 min and again 2-3 ml of TSC agar was overlaid. The test tube was then incubated at 44-45°C for 18-24 h. The black cotton wool like colonies of 2-3 mm in size suspected for *C. perfringens* were taken out with the help of a loop preferably from subsurface and incubated at 37°C in thioglycollate broth for 24 h and the organisms were subjected to biochemical tests for confirmation. Suspected isolates of *C. perfringens* were identified on the basis of grams staining, hanging drop motility test, gelatin liquefaction and nitrate reduction as per standard procedure recommended by Cowan and steel (1974). The isolates were also subjected to lecithinase activity on egg yolk agar and for haemolysis on blood agar.

Lecithinase Activity (Nagler Test): Lecithinase production was detected on egg yolk agar plate by spot inoculation according to method of Willis and Hobbs (1958). Overnight grown culture of *C. perfringens* in thioglycollate broth was inoculated on egg yolk agar plate. It was followed by 24 h incubation under anaerobic condition at 37°C. Plates were observed for zone of opalescence around colonies indicating positive result.

Antibiogram assay: *In-vitro* antibiogram study was carried out on 30 isolates of *C. perfringens*. The assay was performed by disc diffusion technique as per procedure described by Bauer *et al.* (1966). The antibiotics used in this study include ciprofloxacin, enrofloxacin, chloramphenicol, oxytetracycline, cotrimoxazole, cefatoxime, gentamicin, ampicillin, colistin, streptomycin and neomycin. The overnight grown culture of *C. perfringens* in thioglycollate broth were spread on the surface of Mueller Hinton agar surface with the help of sterile cotton swab and after drying, antibiotic discs were placed and the plates were then incubated under anaerobic condition for 24 h at 37°C. The sensitivity/resistance of the cultures was measured by measuring the zone of inhibition around the disc and results were analysed as sensitive or resistant according to manufacturer's (Hi-Media) instructions.

RESULTS AND DISCUSSION

A total of 68 samples of intestinal contents from the enteritis affected broiler birds brought to D.I.Lab, Hisar were screened for isolation of *C. perfringens*. Black wool like colonies of 2-3 mm in size was obtained in TSC agar tubes after enrichment in thioglycolate broth. The organisms in these colonies were confirmed as Gram positive, non-motile, rod shaped bacteria with square ends. The *C. perfringens* isolates were further characterized by biochemical tests. All the isolates were found to be positive for gelatin liquefaction and nitrate reduction. In hemolytic positive cases, hemolysis was seen around the colonies and 41 isolates (83.67%) showed the expression of haemolysin. Haemolysin and lecithinase negative variants of *C. perfringens* isolates were observed in our study. The results of isolation revealed an overall positivity of 72.05% as isolation could be made from 49 samples. Previous studies have reported varied incidence of *C. perfringens* in poultry as 82 to 84% as reported by Miwa *et al.* (1996). Shane *et al.* (1984) and Craven *et al.* (2001a,b) reported that when intestinal contents of broiler chickens were analyzed for presence of *C. perfringens*, approximately 75% to 95% of broiler chickens were found positive. In present investigation, enrichment with heat treatment at 75°C for 20 min followed by use of selective TSC agar was employed for the isolation of *C. perfringens*. The heat treatment used in this protocol, killed most of the heat sensitive pathogenic and non-pathogenic organisms thereby allowing the growth of *C. perfringens*. The use of enrichment before selective plating has been reported to show better recovery percentage of *C. perfringens*. (Fujisawa *et al.* 2001).

All the 49 isolates of *C. perfringens* when tested for lecithinase activity on egg yolk agar, 44 isolates (89.79%) showed a zone of opalescence around colonies (Fig 1) exhibiting the lecithinase activity in them and five isolates were negative for the same. Out of 49 isolates, 39 were positive both for lacithinase and hemolysin, 5 positive for lacithinase but negative for hemolysin, 2 negative for lacithinase but positive for hemolysin and 3 were negative for both. These observations are in agreement to previous reports where some *C. perfringens* strains were also found

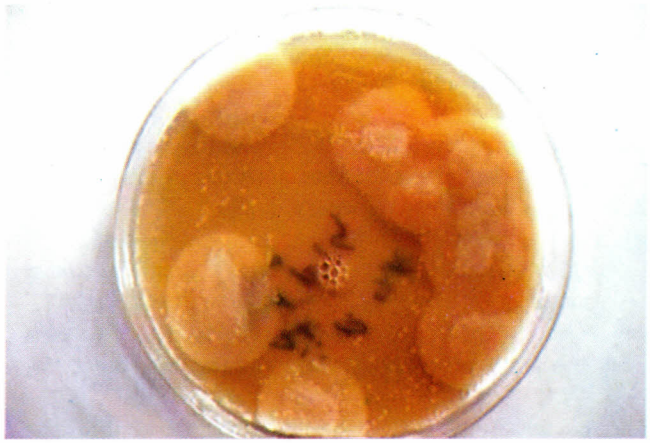

Fig 1. Fried egg appearance of opalescence around colonies of *C. perfringens* on egg yolk agar media.

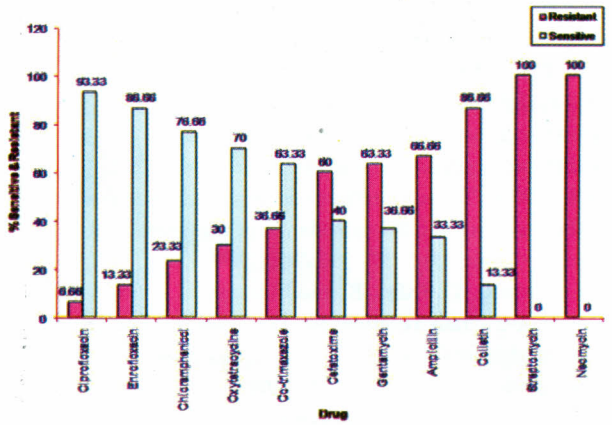

Fig 2. An antibiogram assay result (%) of *C. perfringens* isolates (30).

to be lecithinase negative and non-haemolytic (Skjelkvale, 1979). The biochemical variants of *C. perfringens* have also been reported by Ampratwun (1993) and Skjelvale et al. (1979).

Thirty (30) isolates of *C. perfringens* out of 49 obtained during study were subjected to in-vitro antibiotic sensitivity against eleven commonly used antibiotics. The results of antibiogram assay revealed that *C. perfringens* showed highest sensitivity to fluroquinolones viz. ciprofloxacin (93.3%) and enrofloxacin (86.6%) (Fig 2). The other antibiotics that were found to be sensitive include chloramphenicol (76.6%), oxytetracycline (70%) , co-trimoxazol (63.3%) cefatoxime (40.0%), gentamicin (36.6%), ampicillin (33.3%) and colistin (13.3%). All the isolates were highly resistant to streptomycin and neomycin (100%).

Emergence of resistant strains against antimicrobial agents has become a public health concern all over the world (Witte, 1998). Constant and indiscriminate use of antibiotics in both veterinary and human medicine, especially in developing countries coupled with current knowledge of transfer of antibiotic resistance between various bacteria makes it essential to monitor the susceptibilities of known pathogenic bacteria to antibiotics (Linton, 1977). This study of antibiogram with eleven commonly used antibiotics showed that C. *perfringens* isolates were sensitive to ciprofloxacin, enrofloxacin, chloramphenicol, oxytetracycline, and Co-trimoxazol whereas moderately resistant for cefatoxime, gentamycin, ampicillin, colistin and completely resistant to streptomycin and neomycin.

Neomycin and colistin are very commonly used in combination with other antibiotics as growth promoter in feed which might be responsible for development of complete resistance. These results are similar to antibiotic sensitivity/resistant pattern reported by previous workers, where in *C. perfringens* strains were found to be be sensitive to fluoroquinolones such as ciprofloxacin, enrofloxacin (Ibrahim *et al.*, 2001) and resistant to streptomycin, neomycin and kanamycin (Ibrahim *et al.*, 2001, Secasiu and Pastarnac, 1993).

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