Research Article

Microbiological Studies on *Clostridium perfringens* Isolated from Commercial Poultry of Balochistan

Ramla Achakzai[1,*], Muhammad Kamran Taj[1], Kamran Baseer Achakzai[2]

[1]Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta, PAKISTAN.
[2]Livestock and Dairy Development Department, Balochistan, PAKISTAN.

Submission Date: 22-06-2020; Revision Date: 08-08-2020; Accepted Date: 25-08-2020

ABSTRACT

Necrotic enteritis is one of the most common enteric disease in poultry and a major problem in terms of morbidity and financial cost worldwide. This study was conducted to determine the prevalence of *Clostridium perfringens* in Division of Balochistan, Pakistan.1000 samples were collected from commercial poultry; 68% samples were positive while 32% found negative for *Clostridium perfringens*. Area wise results showed that commercial poultry of Quetta division (19%) was most affected as compare to other divisions of Balochistan, 43.2% of broiler and 24.8% of layer were affected from necrotic enteritis, confirmed through gram staining, biochemical tests and Polymerase Chain Reaction. Polymerase Chain Reaction showed clear bands of 541bp of CPE gene. Antibiograms result showed that *Clostridium perfringens* was sensitive against Amoxycillin and Chloramphenicol while resistance to Sulfamethoxazole, Trimethoprim and Kanamycin. It was concluded that necrotic enteritis was caused by *Clostridium perfringens* that can be eradicated by treating with antibiotic *Penicillin*.

Key words: *Clostridium perfringens*, Commercial Poultry, Balochistan.

Correspondence:
Ramla Achakzai
Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta, PAKISTAN.

Phone no: 00923368028143
Email: ramlacasvab@gmail.com

INTRODUCTION

In Pakistan poultry is one of the central and an active sector of agriculture with a substantial influence on the national GDP. Commercial poultry farming started in Pakistan during early 1960s.[1] One of the most active and disciplined sectors in country is chicken production that contributed about 26.8% in overall meat production, 5.76% to agricultural area and 1.40% to total national GDP during 2016-17; however, its influence in agriculture and livestock worth additional raised at 7.1% and 12.2%, respectively. In previous few years, this sector shown an outstanding growth and developed as a source of employment for about 30 to 35 million from rural population of Pakistan in this very sub-sector of agriculture.[2]

SCAN QR CODE TO VIEW ONLINE

www.ajbls.org

DOI : 10.5530/ajbls.2020.9.31

During last decade, poultry industry growth maximized due to multiplicative qualities and the practice of poultry as a major source of animal protein.[3] Due to poor performance, enteric disorders in poultry are mostly attended with high economic losses, increased mortality rates and medication costs. Several factors such as poor management, nutrition and numerous pathogens are possibly cause of enteric disorders or involve with non-contagious facilities much as feedstuff and supervision related factors.[4]

Necrotic Enteritis (NE) is caused by *C. perfringens*,[5] which is one of the most central enteric diseases in poultry.[6] It is a non-motile, anaerobic, encapsulated, endospore forming, gram positive bacilli bacteria and is commonly found in the intestines of animals and humans. Causing agent of gangrene, gastrointestinal disease in human and enterotoxemic disease in other animals. Its strain produces different toxins and enzyme which induces a specific syndrome.[7]

Enteritis negatively effects the combined system of poultry at high levels.[8] Necrotic enteritis in poultry is associated with influencing factors,[9] including

C. perfringens types A or C. The organisms replicate in gastrointestinal tract (GIT) and produces α toxin that led to mucosal necrosis[10] and it is the main virulence factor.[11]

The *C. perfringens* is resistant to many antibiotics and this resistance could be due to the extensive use of antibiotics. Therefore, this study might help microbiologists to understand the different bacteriological patterns of *C. perfringens*.

MATERIALS AND METHODS

Collection of Samples

A total 1000 gastrointestinal tract and fecal samples were collected from commercial chickens (broiler and layer) of various commercial poultry facilities of Balochistan. Samples were collected aseptically in sterile contamination free polyethylene sachets; and then transported in cold box for further laboratory process at CASVAB, University of Balochistan for microbiological analysis.

Isolation and Identification

Samples were poured in Reinforced Clostridial Medium (RCM) broth and placed in water bath for 10-15 min at 80°C (heat shock) to kill non-spore forming bacteria. The tubes were incubated at 37°C in anaerobic jar for 24-48 hr. Next day culture from tube were taken and streaked on RCM plates and incubate at 37°C for 24 hr. The Colony morphology suggestive for *C. perfringens* were identified by gram staining, biochemical tests (Indole, Methyl red, Voges Proskauer, Citrate utilization test (IMVIC), Sugar fermentation test, Catalase test, Oxidase test, Stormy milk test, Gelatin liquefication test, Lecithenase test) and PCR confirmation was also done.

Growth Characteristics of *C. perfringens* on Different Growth Media

The organism was cultivated anaerobically at 37°C for 24 hr for cultural characteristics study on variety of growth media such as Nutrient agar, Brain Heart Infusion (BHI) agar, Thioglycolate agar, Egg Meat agar and RCM agar.[12]

Effect of Different Growth Media on *C. perfringens*

The presumptive detection of bacteria was carried out on various growth media like Nutrient broth, BHI broth, Egg Meat broth, Thioglycolate broth and RCM broth to observe the effect of organism on different growth media.

Molecular Detection of *C. perfringens*

PCR was used for colonies which were identified as *C. perfringens*. Entire genomic DNA was extracted from samples using genomic DNA purification kit (Thermo scientific, EU). In this study primers of the following arrangement (Forward primer: 5` ACT GCA ACT ACT ACT CAT ACT GTG 3`; Reverse primer: 5` CTG GTG CCT TAA TAG AAA GAC TCC 3`) were used, that amplified a 541bp portion of CPE gene of identified isolate. PCR was performed in 25µl mixture containing 10.5µl grade water, 1µl each set of primer (forward and reverse), 10.5µl master mix (2x AmpMasterTM Tag) and 2µl of DNA template. Samples were subjected to the following thermocycling process in 94°C for 5 min followed by 25 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. PCR product was run on agarose gel (1.5%) and bands were visualized under UV light.[13]

Antibiogram of *C. perfringens*

Antibiotic sensitivity test was performed by modified Kirby-Bauer sensitivity testing technique. Isolates were considered as resistant, intermediate and sensitive to an antimicrobial agent based on inhibitory zone.[14]

RESULTS

Total 1000 samples were collected from different commercial poultry facilities of Balochistan, among which 68% were positive for *C. perfringens* while 32% were negative as shown in Figure 1.

As for as broilers and layers were concerned, 43.2% of broiler and 24.8% of layer were found affected from necrotic enteritis as shown in Figure 2.

The area wise result reveled that, 19% samples were positive from Quetta division, 16% from Kalat division, 11% from Zhob division, 8% from Sibi division, 8%

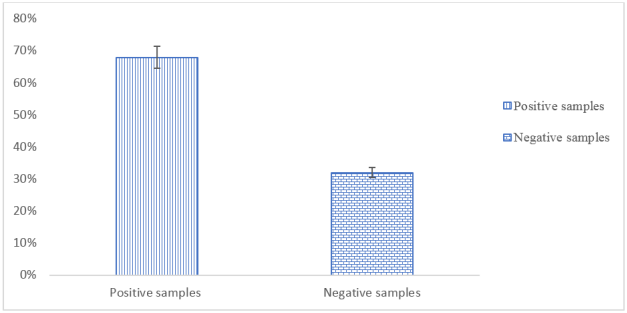

Figure 1: Positive and negative samples of *C. perfringens* isolated from intestinal and fecal samples of commercial poultry of Balochistan.

from Naseerabad division and 6% were positive from Makran division as shown in Figure 3.

Confirmation through Biochemical tests

The present study was conducted to identify the causal agent of necrotic enteritis, which has been affecting commercial poultry of Balochistan and causing heavy economic losses.

Routine methods of bacterial cultures in different media, specific colony characters, staining techniques, microscopic examination and different types of biochemical tests (IMVIC, sugar fermentation, catalase, oxidase, gelatin liquefaction, stormy milk and lecithinase) were used for the isolation and identification of *C. perfringens* as shown in the Table 1.

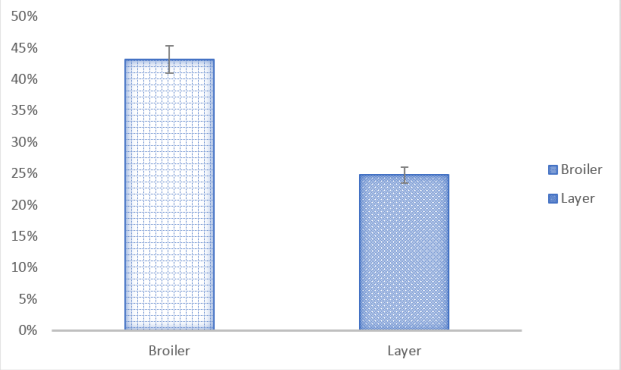

Figure 2: Necrotic enteritis in broiler and layer samples of commercial poultry of Balochistan.

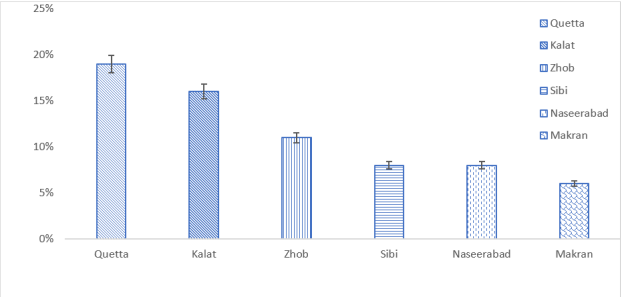

Figure 3: Division wise distribution of positive samples in Balochistan, affected with necrotic enteritis (NE).

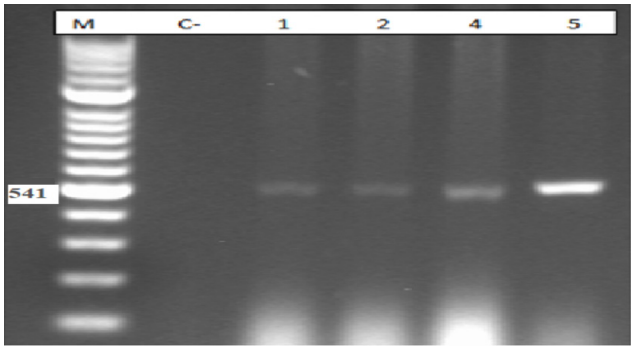

Figure 4: M=DNA leader C= negative control 1, 2,3,4,5 =Samples

Effect of Different Growth Media on *C. perfringens*

It was observed that inoculum in each media showed different growth effects after incubation anaerobically at 37°C for 24 hr. Their quantitative analysis showed that inoculated organism yielded 5.5g on RCM broth whereas on nutrient broth the rise was 1g. The detail about other media are given below (Table 2).

Molecular Identification of *C. perfringens*

In the present study, molecular finding based on gene specific polymerase chain reaction assay was practice in detecting *C. perfringens*. All the isolated of *C. perfringens*

Table 1: Colony morphology on different growth media, gram staining and biochemical tests for identification of *C. perfringens* isolated from commercial poultry of Balochistan.

Media	Confirmation of *Clostridium perfringens*	
Nutrient Agar	1mm White color flat rough-edged colonies	
BHI Agar	2-3mm yellowish flat round colonies	
Thioglycolate Agar	3-3mm Yellowish color round raised colonies	
Egg Meat Agar	2-3mm opaque flat irregular colonies	
RCM Agar	3-5mm Yellowish color round raised colonies	
Gram Staining	Gram positive, Rods, Endospore (oval and sub terminal)	
IMVIC	Indole	Negative
	Methyl Red	Negative
	Voges Proskauer	Positive
	Citrate Utilization	Positive
Sugar Fermentation Tests	Glucose	Positive
	Dextrose	Positive
	Mannitol	Negative
	Sucrose	Positive
	Mannose	Positive
	Maltose	Positive
	Xylose	Positive
Oxidase Test		Negative
Catalase Test		Negative
Urease Test		Negative
Gelatin Liquification Test		Positive
Motility Test		Negative
H2S gas production Test		Positive
Stormy Milk Test		Positive
Lecithenase Test		Positive

Table 2: Effect of Different Growth Media on *Clostridium perfringens*.

Different media	Before Incubation	Temperature	Condition	After Incubation (24 hr.)	Increased (After 24 hr.)
Nutrient Broth	320.8g	37 ˚C	Anaerobic	321.8g	1g
BHI Broth	311.5g	37 ˚C	Anaerobic	315.1g	3.6g
Egg Meat Broth	320.5g	37 ˚C	Anaerobic	323.2g	2.7g
Thioglycolate Broth	325.7g	37 ˚C	Anaerobic	331.1g	5.4g
RCM Broth	320.4g	37 ˚C	Anaerobic	325.9g	5.5g

Table 3: Antimicrobial susceptibility of *C. perfringens* isolated from commercial poultry of Balochistan.

Antibiotic Classes	Antibiotics	Abbreviation	Disc Concentration	Zone of Inhibition (mm)	Antibiogram susceptibility
Chloramphenicol	Chloramphenicol	C	30µgs	26	Sensitive
Penicillin	Amoxycillin	AML	10µgs	28	Sensitive
Tetracycline	Tetracycline	TE	30µgs	16	Intermediate
Glycopeptide	Vancomycin	VA	30µgs	20	Sensitive
Fluoroquinolone	Ciprofloxacin	CIP	5µgs	14	Intermediate
Aminoglycosides	Kanamycin	K	30µgs	00	Resistant
Sulfonamides	Sulfamethoxazole	SXT	25µgs	00	Resistant
	Trimethoprim	W	5µgs	00	Resistant
Polypeptide	Colistine sulphate	CT	30µgs	00	Resistant
	Bacitracin	BC	0.04U	00	Resistant

used in present study produced the predicted size of 541bp amplicon CPE gene as shown in Figure 4.

Antibiogram Sensitivity Test

The *C. perfringens* was sensitive to amoxycillin (28mm), chloramphenicol (26mm), vancomycin (20mm), while intermediate to tetracycline (16mm), ciprofloxacin (14mm) and resistant to (bacitracin, colistine sulphate), (sulfamethoxazole, trimethoprim), (kanamycin) antibiotics as shown in Table 3.

DISCUSSION

C. perfringens is one of the causative agents of necrotic enteritis reported in most areas of the world and badly affects the unified system of poultry production. In present study, isolates from commercial poultry were obtained from cultured samples by picking colonies from each of 1000 samples, in which 68% were positive for *C. perfringens* while 32% were found negative. Risk factor associated for the development of necrotic enteritis was intestinal environment that favors the growth of *C. perfringens*. In commercial poultry the percentage of affective-ness were 43.2% in broiler and 24.8% in layer birds, respectively from necrotic enteritis. Prevalence of *C. perfringens* in different administrative divisions of Balochistan were 19% in Quetta, 16% in Kalat, 11% in Zhob, 8% in Sibi, 8% in Naseerabad and 6% in Makran, respectively observed. Identified isolates were *C. perfringens* based on their cultural, morphological and biochemical characteristics; while all morphological characteristics and biochemical tests findings were in-lined,[15] comparative study of *C. perfringens* growth on various media showed that RCM is the best media for its growth. Antibiotic susceptibility test showed different result for *C. perfringens*, all were sensitive to penicillin, chloramphenicol, glycopeptide and resistance to quinolones, sulfonamides and polypeptide.[16] PCR assay was practiced in detecting *C. perfringens*. All the isolates of *C. perfringens* used in present study produced the predicted size of 541bp amplicon CPE gene.[13]

CONCLUSION

Investigation of necrotic enteritis in commercial chickens based on this study findings will certainly

help in proper diagnosis of the disease, which causes considerable economic loss to the poultry farmers. Thus, this study will also alert poultry professionals about the disease and helps to dictate specific medication as well as postulate prevention and control strategies.

ACKNOWLEDGEMENT

The author would like to thank Director CASVAB, UoB for his support.

CONFLICT OF INTEREST

The Authors have declared that no competing interests exist.

ABBREVIATIONS

GDP: Gross Domestic Products; ***C. perfringens:*** *Clostridium perfringen*; **NE:** Necrotic Enteritis; **GIT:** Gastrointestinal Tract; **UV:** Ultraviolet; **DNA:** Deoxyribonucleic Acid; **RCM:** Reinforced Clostridal Medium; **BHI:** Brain Heart Infusion.

REFERENCES

1. Hussain J, Rabbani L, Aslam S, Ahmad HA. An overview of poultry industry in Pakistan. World Poultry Science Journal. 2015;71(4):689-700.
2. Wasti SE, Ahmad I. Economic Survey of Pakistan. Ministry of Finance, Government of Pakistan. 2017;1(1):19-40.
3. Godfray HC, Beddington JR, Crute IR, Haddad L, Lawrance D. Food security the challenge of feeding 9 billion people. Science. 2010;327(5967):812-8.
4. Hafez HM. Enteric diseases of poultry with special attention to *Clostridium perfringens*. Pakistan Veterinary Journal. 2011;31(3):175-84.
5. Cooper KK, Songer JG. Necrotic enteritis in chickens: A paradigm of enteric infection by *Clostridium perfringens* type A. Anaerobe. 2009;15(1-2):55-60.
6. Shojadoost B, Vince AR, Prescott JF. The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*. Veterinary Research. 2012;43(1):74.
7. Petit L, Gilbert M, Popoff MR. *Clostridium perfringens*: Toxinotype and genotype. Trends in Microbiology. 1999;7(3):104-10.
8. Tschirdewahn B, Notermans S, Wernars K, Untermann F. The presence of enterotoxigenic *Clostridium perfringens* strains in feaces of various animals. International Journal of Food Microbiology. 1991;14(2):175-8.
9. Elwinger K, Schneitz C, Berndtson E, Fossum O, Teglof B, Engstrom B. Factors affecting the incidence of necrotic enteritis, caecal carriage of *Clostridium perfringens* and bird performance in broiler chicks. Acta Veterinaria Scandinavia 1992;33(4):369-78.
10. Ficken MD, Wages PD. Necrotic enteritis diseases of Poultry. Iowa State Press, Ames. 1997;1(10):261-4.
11. Sheedy SA, Ingham AB, Rood JI, Moore RJ. Highly conserved alpha-toxin sequences of avian isolates of *Clostridium perfringens*. Journal of Clinical Microbiology. 2004;42(3):1345-7.
12. Javed S, Rafeeq M, Tariq MM, Rashid N, Ali M. Study on in vitro Biochemical Growth Characterization and Assessment of Hemolytic Toxin of *Clostridium perfringens* Type B and D. Pakistan Journal of Zoology. 2012; 44(6):1575-80.
13. Abd El, Hamid HS, Ellakany HF, Bekhit AA, Elbestawy AR, Bedaway S. Pathogenicity and antibiotic sensitivity of *Clostridium perfringens* isolates from chickens in El Behera governorate, Egypt. Life Science Journal. 2015;12(5):175-82.
14. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic Susceptibility Testing by a Standardized Single Disk Method. American Journal of Clinical Pathology. 1966;45(1):493-6.
15. Miah MS, Asaduzzaman M, Sufian MA, Hossain MM. Isolation of *Clostridium perfringens*, Causal agents of necrotic enteritis in chickens. Journal of Bangladesh Agriculture University. 2011;9(1):97-102.
16. Alimolaei M, Ezatkhah M, Bafti MS, Amini M. Antibiotic susceptibility of *Clostridium perfringens* from organic broiler chickens. Online Journal of Veterinary Research. 2015;19(7):465-70.

Cite this article: Achakzai R, Taj MK, Achakzai KB. Microbiological Studies on *Clostridium perfringens* Isolated from Commercial Poultry of Balochistan. Asian J Biol Life Sci. 2020;9(2):204-8.