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Isolation, identification and molecular characterization of *Clostridium perfringens* from poultry in Kashmir valley, India

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Abstract
Clostridium perfringens, a Gram positive spore forming anaerobic bacteria is associated with necrotic enteritis, necrotic dermatitis, cholangiohepatitis in poultry causing huge economic losses to the poultry Industry. Moreover, *C. perfringens* has been associated with food poisoning in humans. A study was conducted (July 2015 to July 2016) to isolate and characterize *C. perfringens* from apparently healthy and clinically affected poultry of Govt. poultry farms of Hariparbat, Mattan and Instructional Poultry Farm, Shuhama. Samples were also taken from private poultry farms of district Ganderbal, Srinagar and Budgam and Division of Veterinary Pathology, FVSc & AH, Shuhama that were received for post-mortem examination. A total of 224 samples (chicken-184, turkey-29 and duck-11) comprising of feacal material and intestinal contents were collected. A total of 63 (28.12%) isolates (chicken-51, turkey-9 and duck-3) of *C. perfringens* were obtained. Highest (33.63%) occurrence of *C. perfringens* was observed in 2-6 weeks old broilers than the older layer birds (18.91%). All the isolates were found to be *C. perfringens* type A by a multiplex PCR assay for *cpa, cpb, cpb2, etx, cpi,* and *cpe* genes. In addition to *cpa* gene, 9 (17.64%) isolates from chicken also carried *cpb2* gene. None of the isolates carried *cpe* gene. It is thus concluded that *Clostridium perfringens* is prevalent in poultry in Kashmir. Thus, could be associated with causation of disease in them. The presence of *Clostridium perfringens* in healthy poultry birds draws attention towards their presence as reservoir of clostridial infection, which may lead to colossal lossed to the poultry industry.

Keywords: *Clostridium perfringens*, cpa, cpb2

1. Introduction
Poultry production in India has taken a dramatic leap in the last four decades, emerging from an unscientific farming practice to commercial production system with state-of-the-art technological interventions. Enteric diseases are one of the most important problems in the poultry industry leading to production losses, high mortality and risk of contamination of poultry products for human consumption [1]. Enteric disorders are frequently associated with an overgrowth of *Clostridium perfringens,* a Gram positive spore forming anaerobic bacterium. Infections with this bacterium in poultry can cause necrotic enteritis (NE), necrotic dermatitis, cholangiohepatitis, as well as gizzard erosion [2]. Avian necrotic enteritis is caused by particular strains of *C. perfringens* and costs the global poultry industry in excess of $US 2 billion per year in production losses and control measures [3, 4]. Besides, *C. perfringens* is one of the most frequently isolated bacterial pathogens in foodborne ailment episodes in humans [5, 6, 7]. *C. perfringens* type A isolates are also associated with food poisonings in humans [6, 8]. It is considered to be the third most common cause of foodborne illness in the world [9] and annually ranks as one of the most widely recognized causes of food poisoning in the industrialized world [10, 11]. Different meats, including poultry meat, have frequently been reported as the most well-known nourishment vehicles [12, 13].
The virulence of *C. perfringens* is attributable to at least 17 different toxins, while individual strains only produce a subset of these toxins [14, 15, 16]. *C. perfringens* strains are classified into five toxinotypes, A to E according to the production of four major extracellular toxins namely alpha (α), beta (β), epsilon (ε) and iota (ι), while various strains can also produce other toxins including β2, theta(θ) (perfringolysin O), kappa(κ), delta(δ), mu(μ), enterotoxin, necrotic enteritis B-like toxin (NetB), TpeL (toxin *C. perfringens* large cytotoxin) [15, 17]. Type A produces α toxin, type B produces α, β and ε toxins, type C produces α and β toxins, type D produces α and ε toxins and type E produces α and ι toxins.

Keeping in view the above mentioned facts, present study was designed to isolate and characterize *C. perfringens* from apparently healthy and clinically affected poultry reared in organized and unorganized poultry farms of the central Kashmir, India.

2. Materials and Methods

2.1 Collection of samples and isolation of *C. perfringens*

A total of 224 samples comprising of feacal material and intestinal contents, were collected from 184 chickens, 29 turkeys and 11 ducks from organized and unorganized poultry farms, from July 2015 to July 2016.. Out of 184 chicken samples, 118 samples were collected from healthy birds of Govt. Poultry Farm (GPF) Hariparbat (51); GPF, Mattan (46) and Instructional Poultry Farm (IPF), FVSc & AH, Shuhama (21); the remaining 66 samples included samples from healthy chicken (28) of private owners of district Ganderbal, Srinagar and Budgam and Division of Veterinary Pathology (DVP), FVSc & AH, Shuhama (38) that were received for post-mortem examination. 110 and 74 chicken samples were from birds of 2-6 weeks broilers and more than 9 weeks old layers, respectively. The turkey samples were from adult healthy birds of GPF, Hariparbat, while the duck samples were from local backyard farms. The samples were collected in sterile vials and transported to the laboratory on ice. Then the samples were processed immediately for isolation and identification of *C. perfringens*.

2.2 Isolation of *C. perfringens*

For isolation of *C. perfringens,* samples were inoculated in cooked meat medium (Becton, Dickinson and Company, USA) and incubated anaerobically at 37°C for 24 hrs in an anaerobic jar (Oxoid Limited, Thermo Fisher Scientific Inc., UK) containing GasPak™ (Becton Dickinson and Company, USA). Enriched samples were streaked on Sulphite Polymixin Sulphadiazine (SPS) agar plates (Hi-Media Laboratories, Mumbai) and were incubated anaerobically as above. Suspected colonies were stained with Gram's stain and sub-cultured on brain heart infusion (BHI) agar plates until they were free from contaminating bacteria. The pure colonies, suggestive of *C. perfringens* were further streaked on the 5% sheep blood agar and egg yolk agar plates and incubated anaerobically for 24 hr. The colonies producing characteristic double zone of hemolysis around them on blood agar and producing zone of opalescence around the colonies on egg yolk agar were tentatively identified as *C. perfringens*. The pure cultures of *C. perfringens* were preserved at -80°C as glycerol stock (25% glycerol in BHI broth) for further use.

2.3 Extraction of DNA from suspected colonies and molecular typing of isolates

Two to three suspected pure colonies from agar plates were suspended in 100 µl of distilled water in a 1.5 ml microcentrifuge tube. DNA was isolated by boiling method [18].

The isolates were confirmed by detection of *C. perfringens* specific 16S rRNA gene sequences as described by Tonooka *et al.* [19]. For typing, all the *C. perfringens* isolates were screened for six different toxin genes [α (*cpa*), β (*cpb*), ε(*etx*), ί(*cpi*), β2 (*cpb*-2) and enterotoxin (*cpe*)] using a multiplex PCR (m-PCR) described by Van Asten *et al.* [20]. Details of the primers are given in Table 1.

All the PCR assays in this study were performed in 25 µl reaction volume in Mastercycler gradient (Eppendorf AG, Germany). The PCR conditions consisted of initial denaturation at 95 ºC for 15 min, followed by 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 53 ºC for 90 sec and extension at 72 ºC for 90 sec. This was followed by final extension at 72 ºC for 10 min. The MTCC culture of *C. perfringens* type B was used as positive control [19, 20] and visualization by gel electrophoresis.

3. Results

The present study was envisaged with the aim to study the occurrence, identification, characterization and molecular diversity of *Clostridium perfringens* from different species of poultry of Kashmir valley.

The fecal and morbid samples after overnight incubation in cooked meat medium, showed turbidity and pink colouration of the meat pieces (Figure 1). On overnight incubation of the inoculum from cooked meat medium, colonies appeared typically black pigmented or creamish in colour on Sulphite Polymixin Sulphadiazine (SPS) agar suggestive of *C. perfringens*. Suspected colonies of *C. perfringens* were further subcultured on the SPS agar plates to obtain pure culture (Figure 2). On sheep blood agar the isolates produced double zone of hemolysis around the colonies characteristic of *C. perfringens*. Interestingly, the inner zone of complete hemolysis is caused due to theta toxin while outer zone of partial hemolysis is caused due to phospholipase activity of alpha toxin (Figure 3).

On Gram's staining the black colonies isolated on SPS agar, Gram-positive rods appeared suggestive of *C. perfringens* (Figure 4). On capsular staining the capsules appeared as faint halos around dark cells of *C. perfringens* (Figure 5). On inoculation over egg yolk agar plates the *C. perfringens* produced opalescence around the colonies indicating the lecithinase activity which is characteristic of the organism as depicted in Figure 6.

Out of 184 samples from chicken, *C. perfringens* was isolated from 51 (27.71%) samples. Similarly, 9 (31.03%) and 3 (27.2%) samples yielded *C. perfringens* from 29 turkey and 11 duck samples, respectively. Occurrence of *C. perfringens* in chicken was higher (33.63%) in 2-6 weeks old broilers than the layer birds (18.91%). Details of the isolates from different sources are given in Table 2.

Out of a total of 51 isolates from chicken, all the 51 isolates were found to possess *cpa* gene alone as a major toxin gene, thus were designated as toxinotype A. None of the isolates carried *cpb*, *etx* or *cpi* genes indicating the absence of *C. perfringens* toxinotype B, C, D or E in chicken samples.

4. Discussion

Clostridium perfringens is a ubiquitous bacterium found in poultry houses and its surroundings, including water, poultry faeces, feed, soil and air [21], as well as in a wide variety of raw and processed foods particularly meat and poultry [22]. *C. perfringens* is believed to be the cause of variety of economically significant diseases in domestic animals [14].

Genetically *C. perfringens* is a diverse organism and has successfully been isolated from the GIT of animals and humans. Particularly in poultry, *C. perfringens* is present throughout almost the full length of the GIT; it has been isolated from the crop, gizzard and caeca [23, 24]. *C. perfringens* toxinotypes are responsible for varied disease syndromes in livestock animals and poultry. In the present study healthy as well as suspected poultry populations from different regions of Kashmir valley were screened for the presence of *C. perfringens* toxinotypes.

The features of the colonial morphology, which included, appearance of blackish colonies which is due to reduction of sulfite to sulfide by *C. perfringens* which in turn reacts with

iron and forms a black iron sulfide precipitate and glistening round colonies surrounded by inner zone of complete haemolysis and an outer zone of discoloration and incomplete haemolysis on sheep blood agar after 48 h of incubation at 37 ºC under anaerobic condition, is concurred with earlier observation by other authors [21, 25]. It was also described that *C. perfringens* colonies were circular, flat, greyish and surrounded by zone of double hemolysis after cultured anaerobically on blood agar at 37°C for 48 hrs [26]. Microscopically all isolates were Gram-positive spore-forming bacilli, biochemical identification of the isolates showed both catalase and lecithinase positive, these results also stated by Quinn *et al*. [27]. On egg yolk agar, all the isolates attacked lecithin in the medium and produced opalescence around the streak. These findings are in accordance with those of Malmarugan *et al*. [28].

These findings correlate with the previous studies by Lovland and Kaldhusdal [4], where the authors reported detection of C. *perfringens* in 2-6 wk broiler chickens. Wages and Opengart also reported isolation of *C. perfringens* from 7 to 16 wk layer birds [29].

Our findings revealed that 51 (27.71%) of 184 samples from chicken, 9 (31.03%) of 29 samples from turkeys and 3(27.2%) of 11 duck samples were positive for *C. perfringens* based on isolation and PCR amplification of alpha specific gene. The results are in agreement with Osman *et al*. [30] who reported that 17 (35.41%) out of 48 samples from healthy chickens were positive for *C. perfringens.*

Out of the 51 isolates from chicken, 37 (72.54%) were isolated from broilers of 2 to 6 wk of age, 14(27.46%) from layers of above 9 weeks of age. These findings corroborate with studies by Lovland and Kaldhusdal [4], where the authors reported detection of C. *perfringens* in 2-6 wk broiler chickens. Wages and Opengart also reported isolation of *C. perfringens* from 7 to 16 wk layer birds [29].

Clostridium perfringens isolates obtained in this study were screened for presence of six toxin genes viz; *cpa, cpb*, *etx, cpi, cpb2* and *cpe*. All isolates from chicken, turkeys and ducks belonged to type A, none of these isolates possessed *cpb, etx, cpi* toxin gene, indicating absence of *C. perfringens* toxinotype B, C, D, These findings are in agreement with the observations of Nauerby *et al*. [23], Baums *et al.* [31]. Malmarugan *et al*. [28] reported that out of 20 isolates, all indicated the presence of Toxinotype A. The results of the present study are also in accordance with Engstron *et al*. [32] who analysed 53 isolates of *C. perfringens* from poultry of different parts of Sweden by PCR for toxinotyping and reported that all the isolates belonged to Toxinotype A of *C. perfringens.* Park *et al.* [33] observed that all the isolated strains were identified as Toxinotype A using multiplex PCR for toxinotyping from chickens in Korea.

In addition to four major toxin genes, one minor toxin gene *cpb2* gene was detected in 9 isolates of toxinotype A in chicken. In turkeys and ducks, all *C. perfringens* toxinotype A isolates revealed the presence of species specific *cpa* gene but none of the isolates harboured *cpb2* gene. Gholamiandekhordi *et al*. [24] found 5 isolates out of 63 to be β2 toxin-positive, which indicates that the β2 toxin is not an important or essential virulence factor in the development of disease in poultry. Osman *et al*. [30] also stated that the isolates from poultry harboured the *cpb2* gene. Thomas *et al*. [34] also reported the presence of *cpb2* gene among *C. perfringens* toxinotypes in India. In present study, none of the isolates carried *cpe*-gene which is in agreement with the finding of other authors [32, 35].

5. Conclusion

Taking the results of the present study into consideration, it is concluded that *Clostridium perfringens* is prevalent in poultry in Kashmir. Thus, could be associated causation of disease in them. The presence of *Clostridium perfringens* in healthy poultry birds draws attention towards their presence as reservoir of clostridial infection, which may lead to occurrence of disease when in immunologically depilated conditions which may lead to significant economic losses to the poultry industry.

Table 1: Details of primers used for detection and typing of *C. perfringens* isolates

S. No.	Target gene	Primer Sequence (5′-3′)	Primer conc. (µM)	Product size (bp)	Reference
1.	16S RNA	F-TAACCTGCCTCATAGAGT R-TTTCACATCCCACTTAATC	0.4	481	[32]
2	*cpa*	F-GCTAATGTTACTGCCGTTGA R-CCTCTGATACATCGTGTAAG	0.4	324	[20]
3	*cpb*	F–GCGAATATGCTGAATCATCA R–GCAGGAACATTAGTATATCTTC	0.4	195	[20]
4	*etx*	F–TGGGAACTTCGATACAAGCA R–AACTGCACTATAATTTCCTTTTCC	0.4	376	[20]
5	*cpi*	F-AATGGTCCTTTAAATAATCC R-TTAGCAAATGCACTCATATT	0.4	272	[20]
6	*cpb2*	F–AAATATGATCCTAACCAACAA R-CCAAATACTCTAATYGATGC	0.4	548	[20]
7	*cpe*	F-TTCAGTTGGATTTACTTCTG R-TGTCCAGTAGCTGTAATTGT	0.4	485	[20]

Table 2: Details of the *C. perfringens* type A isolates recovered from different sources

Species	Source	No of samples		No of isolates
		Healthy	Diseased	
Chicken	GPF, Hariparbat	51	-	17 (5)
	GPF, Mattan	46	-	14 (2)
	IPF, FVSc & AH, Shuhama	21		5
	Private farms	28	-	5
	DVP, FVSc & AH, Shuhama	-	38	10 (2)
Turkey	GPF, Hariparbat	29	-	9
Duck	Backyard farm	11	-	3
Total		186	38	63

Figures in parenthesis indicate number of *cpb2* positive isolates

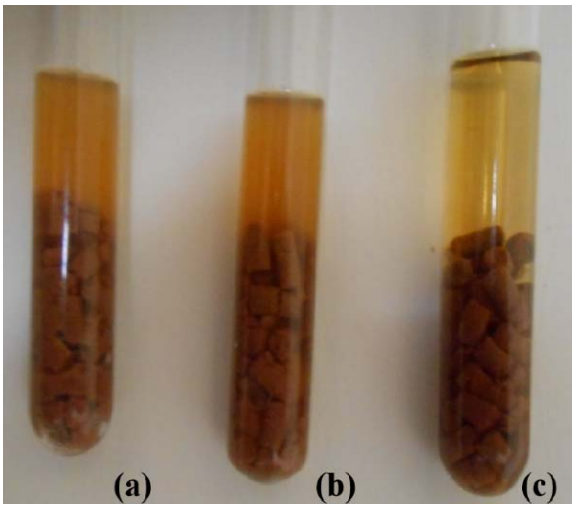

Fig 1: Cooked meat media with *Clostridium perfringens*. Tubes a & b showing 24 hrs anaerobic bacterial growth after inoculation with samples. Tube c showing cooked meat medium before inoculation.

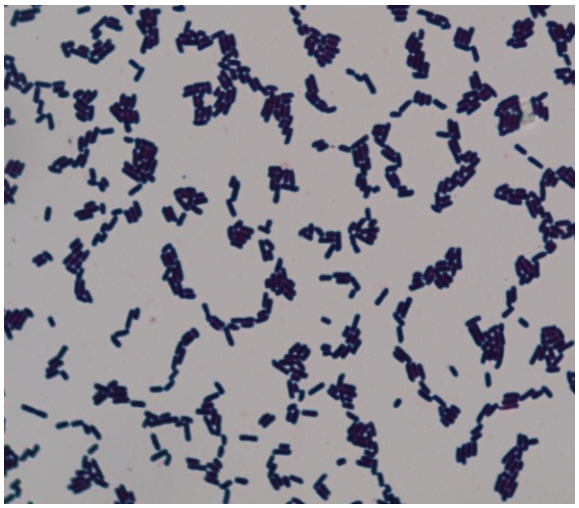

Fig 4: Gram stained smear of pure culture of *Clostridium perfringens* showing Gram positive violet rods under oil immersion (100X).

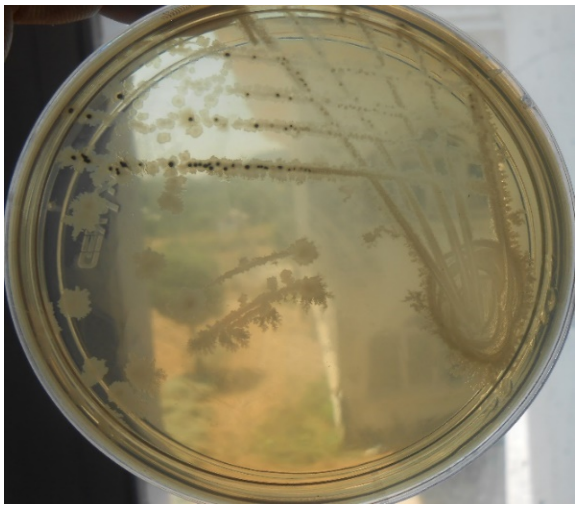

Fig 2: Pure culture of *Clostridium perfringens* on Sulphite Polymixin Sulphadiazine (SPS) agar medium showing black and cream coloured colonies.

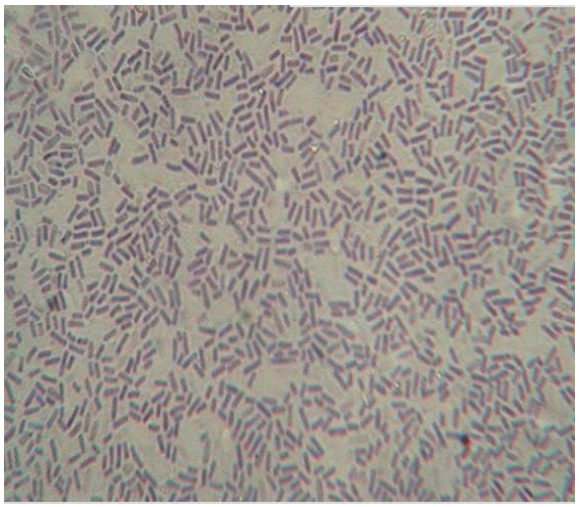

Fig 5: Capsular staining (Anthony's method) of pure culture of *Clostridium perfringens* showing halo around the rods under oil immersion (100X).

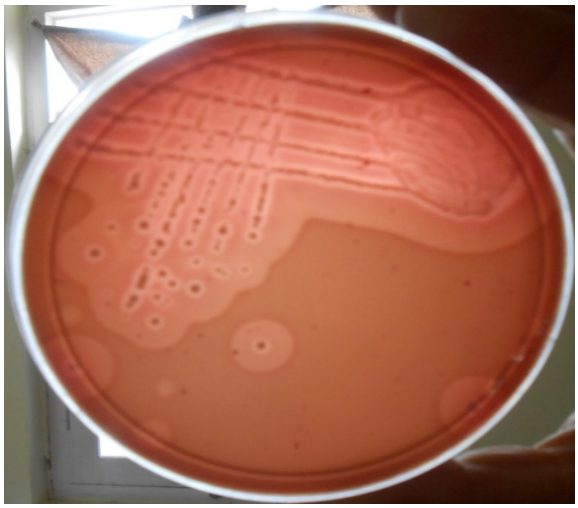

Fig 3: Double zone of hemolysis produced by *Clostridium perfringens* on sheep blood agar Outer zone of incomplete haemolysis &Inner zone of complete haemolysis.

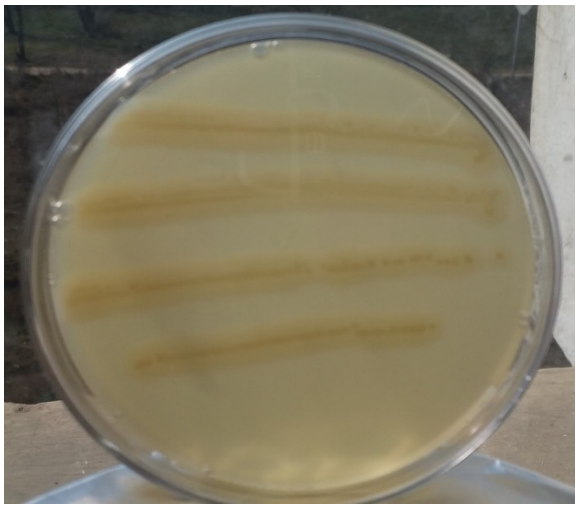

Fig 6: Lecithinase activity of *Clostridium perfringens* on egg yolk agar after 24 hrs of growth. Streaks showing opalescence produced around *Clostridium perfringens* streaks.

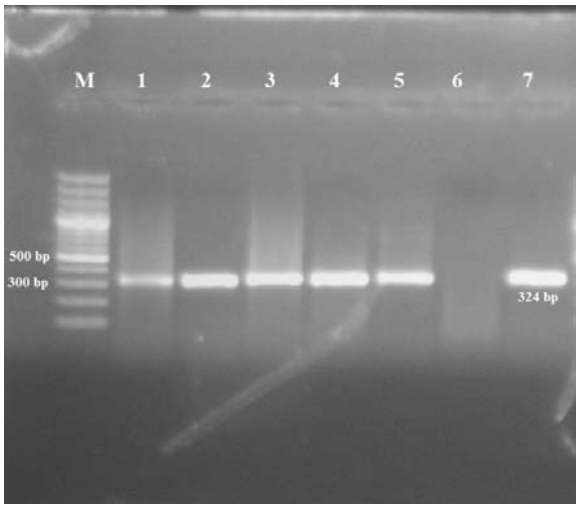

Fig 7: Agarose gel electrophoresis showing 324 bp amplicon of alpha gene of *Clostridium perfringens,* Lane M: 100 bp DNA ladder, Lane 1-5: Isolates positive for *C. perfringens,* Lane 6: Negative control, Lanes 7: Positive control.

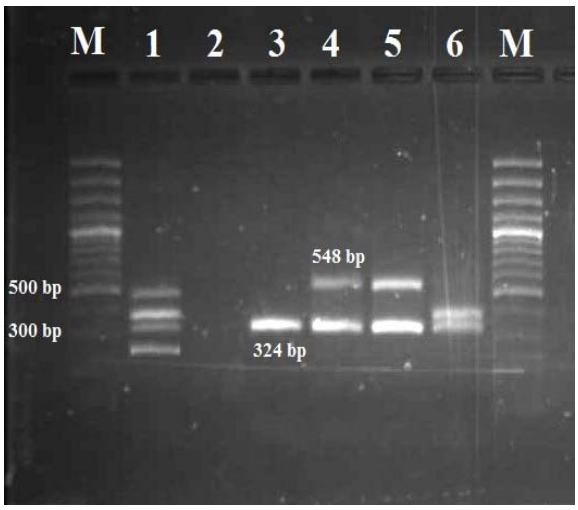

Fig 8: Agarose gel electrophoresis of multiplex PCR amplicons of different virulence genes of *Clostridium perfringens,* Lane M: 100bp DNA ladder, Lane 1: Positive control of *C. perfringens* Type B with amplified *cpa* (324bp), *cpb* (195bp) *etx* (376bp) & *cpe* (485bp) genes, Lane 2: Negative control, Lane 3: *C. perfringens* Type A with amplified *cpa* only, Lane 4-5: *C. perfringens* Type A with amplified *cpa* and *beta2* (548bp) genes, Lane 6: Positive control of *C. perfringens* Type D with amplified *cpa* and *etx* gene.

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