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Isolation, prevalence and molecular toxinotyping of *Clostridium perfringens* from healthy and diseased sheep of Kashmir, India

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Abstract
In the present study, prevalence of *Clostridium perfringens* in the healthy and diseased sheep of Kashmir valley was investigated along with the detection of the various toxinotypes present. A total of One hundred (100) faecal samples of ovine population comprising of those taken from healthy flock (50) and from diarrheic cases (50) of the organized and unorganized sectors of Kashmir valley were collected for screening of *Clostridium perfringens.* On complete isolation and identification, a total of 61 samples were found to be positive for the presence of *C. perfringens* with a prevalence of 42% and 80% from healthy and diarrheic cases respectively. Molecular typing of the isolates by multiplex PCR employing *cpa, cpb, etx,* and *iap* genes revealed 37(60.65%) isolates were Type A, 2 (3.27%) were Type B, 1(1.63%) Type C, and 21(34.42%) were Type D respectively, while no isolate of *C. perfringens* Type E was recovered from any of the samples collected.

Keywords: *Clostridium perfringens*, healthy sheep, diseased sheep, toxinotypes, multiplex PCR

Introduction
Clostridium perfringens belongs to the genus *Clostridia* and being one of its member pathogen has importance in both the veterinary and medical fields. It is a non-motile, spore-former which exists as a Gram-positive rod (0.6-0.8 x 2-4µm) being facultatively anaerobic with oval, sub-terminal spores that bulge from the mother cell. This organism is ubiquitous in environment as it is commonly present in soil, foods, sewage, faeces and the intestines of many healthy humans and animals [10].

This bacterium has emerged as the most common and important pathogen of humans and livestock [10, 16]. *C. perfringens* results in histotoxic infections, like gas gangrene (myonecrosis), anaerobic cellulitis and wound infections in humans [16] along with several diseases which originate from the intestines and typically manifest as enteritis or enterotoxaemia. In domestic animals it frequently causes necrotic enteritis, enterotoxaemia, lamb dysentery, equine colitis and neonatal toxemias [17].

The production of exotoxins by *C. perfringens* is responsible for its pathogenic nature and these exotoxins also form the basis for classifying it into five types, A, B, C,D and E. Type A produces only alpha toxin (α), Type B produces alpha(α), beta(β) and epsilon toxin(ε), Type C produces alpha (α) and beta toxin(β), Type D produces alpha(α) and epsilon toxin(ε) and Type E produces alpha (α)and iota toxin(ι). Type A strains form an important part of the intestinal flora of humans and animals, while types B-E are involved mostly in disease processes of animals [4, 12].

Traditionally, the typing of *C. perfringens* was done employing toxin neutralization test in mice but this method being time consuming and laborious consumes a lot of antisera and experimental animals. However with the advancement of various techniques these methods have been replaced by PCR-based detection [3, 11, 21]. Detection and toxinotyping of *C. perfringens* in a particular area is of prime preference for the development of the appropriate vaccines in the fields. The aim of this study was to investigate prevalence of *C. perfringens* and to type the isolated strains by multiplex PCR from healthy and diseased sheep of Kashmir valley.

2. Materials and Methods

2.1 Reference Bacteria

The current study used the reference strain of *Clostridium perfringens* ATCC (13124) (American Type Cell Culture) procured from Hi-media (Mumbai, India) as a positive control.

2.2 Samples

A total of 100 faecal samples comprising of 50 samples from healthy flock and 50 from the animals with history of diarrhea were collected from different areas of Kashmir valley and transported aseptically in sterile zipper bags to the Division of Veterinary Public Health laboratory, FVSC & A.H, (ALUSTENG-SKUAST-K) in an ice box for its immediate microbiological analysis, isolation, identification and typing of *Clostridium perfringens* isolates. All these samples were processed within 2-3 hrs of the collection.

2.3 Isolation and Identification

C. perfringens was isolated and identified as per the Standard microbiological protocol [5, 6] with suitable modifications. Briefly, all the samples were inoculated into Cooked Meat medium (Hi-media) and incubated at 37 ºC for 24-48 hours using anaerobic environment of 15% CO_2. A loopful culture from turbid Cooked Meat medium was then streaked on Sulfite polymyxin sulfadiazine (SPS) agar for the selective isolation of organism. The petriplates were incubated for 24-48 hours at 37 °C in anaerobic jar (Oxoid, Thermo Fisher Scientific Inc., UK) with GasPak^{TM} Anaerobic Container System (Becton, Dickinson and Company, Sparks, MD, USA). The greyish colonies from SPS agar plates were transferred to SPS agar slants and further later on incubated at 37ºC for 24-48 hours anaerobically and stored at 4ºC for further identification.

2.4 Plating on selective agar

Selective isolation of *C. perfringens*, was performed by agar overlay technique.Shahidi-Ferguson-Perfringens (SFP) agar and Tryptose Sulphite Cycloserine (TSC) agar (Himedia Labs, Mumbai,India) were used as selective media. 20 ml of sterile SFP medium was added into a petriplates on which a loop full of inoculum from FTM or RCMB was streaked allowing it to solidify for 5-10 minutes followed by again overlaying of 10 ml of SFP medium. The petriplate was then incubated at 37 ºC for 24 h. The growth of *C. perfringens* was evident by formation of typical black coloured colonies on SPS agar medium

Additionally a loop full of inoculum from FTM or RCMB was streaked on TSC agar which otherwise contain egg yolk as well. The agar plates were incubated at 37°C for 18-24 hours anaerobically. The formation of yellow gray opaque zone of lecithinase activity was taken as indicative of *C. perfringens* presence [5, 6].

2.5 Morphological and biochemical characterization

Morphologically, the presumptive isolates of *C. perfringens* identified were verified on the basis of grams staining, capsular staining, spore staining, motility, catalase test, triple sugar iron agar test, gelatine liquefaction, nitrate reduction and lactose fermentation. The isolates were further confirmed with double haemolysis on blood agar, lecithinase and proteolytic activity [7, 15].

2.6 Molecular Characterization

Molecular characterization of *C. perfringens* isolates confirmed by morphological and biochemical tests was done using species specific Polymerase Chain Reaction targeting 16S rRNA gene for identifying the species which was followed by toxino-typing of isolates by multiplex PCR.

2.7 Bacterial DNA isolation

The individual suspected colonies from SPS Agar medium were suspended in 1.5ml microcentrifuge tube containing 100µl sterile double distilled water and boiled for 10 min and then kept in crushed ice for 10 min for cooling. After proper thawing the tubes containing the suspension were centrifuged at 6000 rpm for 5 min and the supernatant was collected which served as a DNA template.

2.8 16S rRNA gene amplification

After phenotypic identification of *C. perfringens* isolates, further confirmation of presumptive positive strains was done by employing species-specific PCR targeting 16S rRNA gene of *C. perfringens* using standard protocol as described by Tonooka *et al*. (2005). In brief, the amplification of bacterial DNA for detection of 16SrRNAgene was set up using 25µl reaction volume employing primers given in Table1. The standardized PCR protocol included 2.5 µl of 10X PCR buffer (100 mM Tris–HCl buffer, pH 8.3 containing 500 mM KCl and 0.01% gelatin), 0.2 µl of 25 mM dNTP mix, 2mM MgCl2 and 0.4µM ofeach primer sets, 1 unit of Taq DNA polymerase, a single colony as DNA template and sterilized milliQ water to make up the reaction volume. The cycling conditions for PCR employed initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 49°C for 90 s, and extension at 72°C for 90 s. This was followed by final extension at 72°C for 10 min. Sterile distilled water without DNA template was used as a negative control whereas reference strain of *C. perfringens* ATCC-13124 was used as a standard positive control. The resultant PCR products were stored at -20ºC until further analysis by agarose gel electrophoresis.

2.9 Toxinotyping of *C. perfringens* isolates

Isolates of *C. perfringens* were screened for the presence of toxigenic genes like *cpa*(α-toxin), *cpb*(β-toxin), *etx*(ε-toxin) and *iap*(ι-toxin) respectively by employing multiplex PCR assay as per standard procedures given with some desired modifications [3, 11, 20]. Toxin gene screening was utilized for typing of *C. perfringens*. The primers used for amplification of genes are listed in Table 2.

The multiplex PCR was set up in 25µl reaction volume and the reaction mixture included, 2.5µl of 10X PCR buffer (20mM Tris-HCl buffer, pH 8.4 containing 50 mMKCl), 25µl of25mM $MgCl_2$, 0.3µl of 10mM of each dNTPs, 1 unit of *Taq* DNA polymerase(MBI-Fermentas), 3.0 µl template DNA and 1µl of 10 picomoles the primer each -containing forward and reverse primers, Sterilized milliQ water was used to make up the final reaction volume.

The DNA amplification reaction was performed in Master Cycler Gradient PCR machine (Applied Biosystems-Singapore) with a pre-heated lid. The cycling conditions for PCR included an initial denaturation of DNA at 95°C for 15 minutes, followed by 35 cycles each of 30 seconds denaturation at 94°C, 90 seconds annealing at 53°C and 90 seconds extension at 72°C, followed by a final extension of 10 minutes at 72°C with its hold at 4°C. The resultant PCR

products were stored at –20 °C until further analysis by agarose gel electrophoresis in 1.5% agarose (SRL) stained with ethidium bromide (5µg/ml) with 100bp plus Gene ladder used as a molecular size standard for estimation of amplified products.

3. Results

3.1 Prevalence of *C. perfringens* based on isolation

Complete morphological and biochemical analysis of ovine faecal samples revealed that out of the total 100 samples from healthy (50) and diarrheic cases (50), 61 samples were found to be positive for presence of *C. perfringens* making an overall prevalence of the organism to be 61%. The organism was recovered from 21 of the 50 faecal samples of healthy ovine population with a prevalence percentage of 42. However, the diarrheic cases were found to contain higher levels of the organism as 40 out of the total 50 cases were positive revealing a prevalence of 80%. The highest occurrence of *C. perfringens* was found in samples from the diarrheic cases of unorganized sector (84%) as compared to the organized sector (76%). Likewise samples from the healthy sheep of organized and unorganized sector showed a percent prevalence of 20 and 64, respectively. All the 61 isolates from sheep were morphologically and biochemically identified by Gram staining (Figure 1) capsular staining, lecithinase activity on egg yolk agar media (Figure 2), triple sugar iron (TSI) test, and formation of double zone of hemolysis on 5% sheep blood agar (Figure 3) as *C. perfringens.*

When the Chi square test was applied, the data revealed significant statistical difference ($p<0.005$) between healthy and diarrheic faecal samples with respect to the occurrence of *C. perfringens*. A significant difference ($p<0.005$) was also observed in faecal samples from healthy animals of organized and unorganized sectors, reporting a higher level of prevalence in unorganized sector. However, no significant difference ($p<0.005$) was recorded between diarrheic cases from ovine population of organized and unorganized sectors with respect to the presence of the organism under study as predicted in Table 3.

3.2 Molecular identification and toxinotyping

The genus *Clostridium* is a very diverse group of related organisms therefore, for accurate classification and identification of species Polymerase Chain Reaction targeting 16S rRNA gene is frequently employed. A pair of primers was used for the confirmation of *C. perfringens* and among a total of 100 isolates identified phenotypically, the species specific PCR detected all 100 isolates as *C. perfringens* amplifying a 481bp product (Figure 4).

All the *C. perfringens* isolates obtained from ovine faecal samples were typed into toxinotypes A, B, C, D, and E by employing a multiplex PCR assay simultaneously targeting four major toxin genes *cpa*, *cpb*, *etx*, and *iap*. Under the assigned multiplex PCR condition, six fragments of virulence genes were amplified having the predicted size of 324, 195, 376 and 272bp for *cpa*, *cpb*, *etx* and *iap* genes respectively.

Among a total of 61 isolates recovered during the study, 37 (60.65%) were found to carry *cpa* gene as a major toxin gene and thus were designated as Type A while21(34.42%) isolates harbored both *cpa* and *etx* genes, thus were designated as toxinotype D and 2 (3.27%) isolates carried in addition to *cpa* gene *cpb* and *etx* gene thus were Type B,1 (1.63%) isolate carried both *cpa* and *cpb* gene and hence were classified as Type C, However, during the current study none of the isolates could be identified as Type E. Among 21 isolates from healthy sheep, 16 (76.19%) were found to be toxinotype A, 4 (19.04%) were positive for toxinotype D, while only 1 (4.76%) was identifed as toxinotype C. Out of total 40 isolates from sheep suffering diarrhea, 21 (52.50%) isolates were found to be toxinotype A while 17 (42.5%) isolates were detected as toxinotype D and 2 (5.00%) were toxinotype B as shown in Figure 5.

4. Discussion

C. perfringens is widely dispersed in environment and is also found in the intestines of animals as well as humans, thus it can be pathogenic to both of them. This organism produces disease in sheep as well as goats, most of which are commonly called as *Enterotoxemia* leading to huge economic losses to the farmers [19]. Since it is a normal inhabitant of intestinal tract of animals, whenever there is unfavorable alteration in intestinal environment either by sudden changes in diet or other factors, the organism proliferates in large numbers, produces several potent toxins and hence result in disease. In the present investigation, healthy as well as diarrheic ovine populations from different regions of Kashmir valley were screened for the presence of *C. perfringens.* On complete microbiological isolation, identification using biochemical tests and PCR amplification of 16S rRNA gene, it was inferred that of the total 100 ovine faecal samples, 61 samples (61.00%) were found to be positive for *C. perfringens*. These findings correlate with the study reporting 110 (72.36%) of 152 sheep and 15 (60%) of 25 goats positive for *C. perfringens* based on isolation and PCR amplification of 16S rRNA gene [13]. However, the diarrheic cases were found to contain significantly higher levels of the organism as 40 out of the total 50 cases were positive revealing a prevalence percentage of 80% ($p<0.05$), while in case of healthy ovine population the organism could be recovered from 21 faecal samples predicting a prevalence of only 42%. Prevalence of 77% of *C. perfringens* from diarrhea cases of sheep populations in Giza governors, Egypt [1] and turkey [8]. Among the healthy ovine faecal samples, collected from organized and unorganized sectors, a prevalence of 20% and 64% was observed respectively. Likewise diarrheic cases from these sectors showed a percent prevalence of 76 and 84% respectively pointing towards highest occurrence of *C. perfringens* in unorganised sector irrespective of the health status of the animals. This higher prevalence of the organism in the unorganised sectors may be attributed to lack of proper vaccinations, heavy feeding and grazing on luxurious pastures [8].

C. perfringens on the basis of its ability to produce major lethal toxins (alpha, beta, epsilon, and iota toxins) has been categorized into five toxigenic types (A-E). In the present investigation the isolates were subjected to a multiplex PCR for toxinotyping and it was concluded that out of 61 isolates of *C. perfringens* 37 (60.65%) were Type A, 21(34.42%) isolates were designated as toxinotype D, 2 (3.27%) isolates were Type B,1 (1.63%) isolate was classified as Type C. However, during the current study none of the isolates could be identified as Type E. The findings corroborate with the work Kalender *et al.* (2005) who genotyped 52 *C. perfringens* strains from diseased sheep and reported 33 (64%) isolates as Type A, 11 (21%) Type D and 8 (15%) Type C, thus concluding toxinotype A as the most predominant type in sheep. From a total of 100 *C. perfringens* isolates, 21 were

typed as *C. perfringens* type D commonly causes clostridial enterotoxemia in sheep and goats but is only occasionally found as a causative agent of clostridial enterotoxemia in other animal species. It is one of the most frequently occurring diseases of sheep and goats worldwide with reported prevalence rates ranging between 24.13% and 100% [2, 9].

Among 21 isolates recovered from healthy sheep, 16 (76.19%) were found to be toxinotype A, 4 (19.04%) were positive for toxinotype D, while only 1 (4.76%) was identified as toxinotype C and from a total of 40 isolates from sheep suffering from diarrhea, 21 (52.50%) isolates were found to be toxinotype A while 17 (42.5%) isolates were detected as toxinotype D and 2 (5.00%) were toxinotype B. similar findings were recorded when 114 isolates were toxinotyped by multiplex PCR from healthy sheep and goat of Kashmir region and observed 87.7% as *C. perfringens* type A, while 12.3%were type D [14]. From diarrheic animals 75% and 25% out of 172 *C. perfringens* isolates were type A and type D, respectively.

5. Conclusion

C. perfringens one of the important pathogen has emerged as a significant disease causing organism in both animals and humans, in present study also we screened a total of 100 ovine faecal samples giving an overall prevalence of 61% i. e they were found to be positive for *C. perfringens.* Out of 61 isolates of *C. perfringens* 37 (60.65%) were Type A, 21(34.42%) isolates were designated as toxinotype D, 2 (3.27%) isolates were Type B, 1 (1.63%) isolate was classified as Type C. However, none of the isolates could be identified as Type E. However, irrespective of the health status of animal Type A was prevalent to higher degree followed by Type D. Thus indicating animals being an important source of *C. perfringens* Type A to humans and food.

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Table 1: Primer Sequence used for 16S *rRNA*gene

Target gene	Primer Sequence (5'→3')	Product size (bp)	Reference
16SrRNA	Forward-TAACCTGCCTCATAGAGT Reverse-TTTCACATCCCACTTAATC	481	[18]

Table 2: Primers used for Toxinotyping of *C. perfringens*

S. No	Gene	Primer Sequence(5' -3')	Product size(bp)	Reference
1.	*Cpa*	F- GCTAATGTTACTGCCGTTGA R- CCTCTGATACATCGTGTAAG	324	[3, 11, 20]
2.	*Cpb*	F- GCGAATATGCTGAATCATCTA R- GCAGGAACATTAGTATATCTTC	195	
3.	*etx*	F- TGGGAACTTCGATACAAGCA R- AACTGCACTATAATTTCCTTTTCC	376	
4.	*Iap*	F- AATGGTCCTTTAAATAATCC R- TTAGCAAATGCACTCATATT	272	

Table 3: Prevalence of *C. perfringens* in Ovine faecal samples

Nature of Sample	Source of Sample		No of Samples screened	No. Positive for *C. perfringens* (%)	Total percent positive for *C. perfringens*	χ^2 (p- value)	
Ovine Faeces	Healthy	Organised sector	25	5 (20)	42	9.934 (0.002)	25.68 (0.001)
		Unorganised sector	25	16 (64)			
	Diarrhoeic	Organised sector	25	19 (76)	80	0.5 (0.480)	
		Unorganised sector	25	21 (84)			
Total			100	61	61		

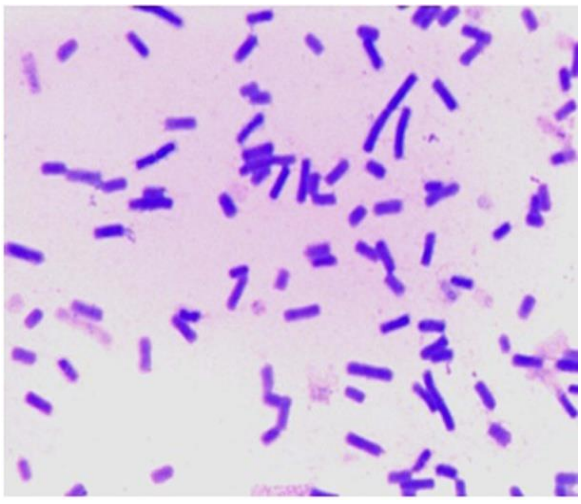


Fig 1: Gram staining of *Clostridium perfringens* depicting large Gram positive rods under oil immersion (100X)

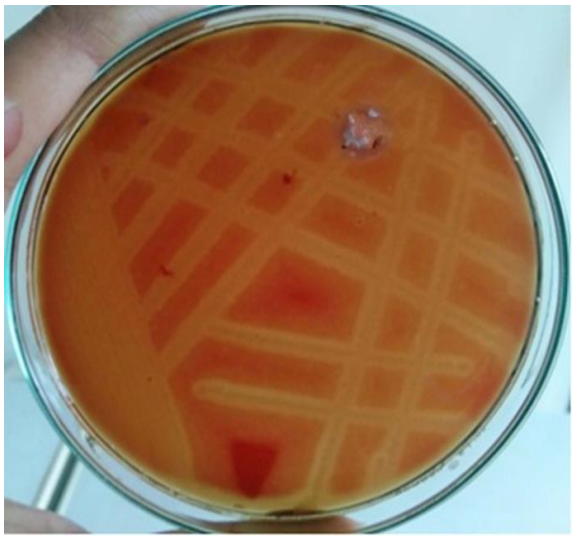


Fig 2: β- haemolysis produced by *Clostridium perfringens Type* A on 5% sheep blood agar

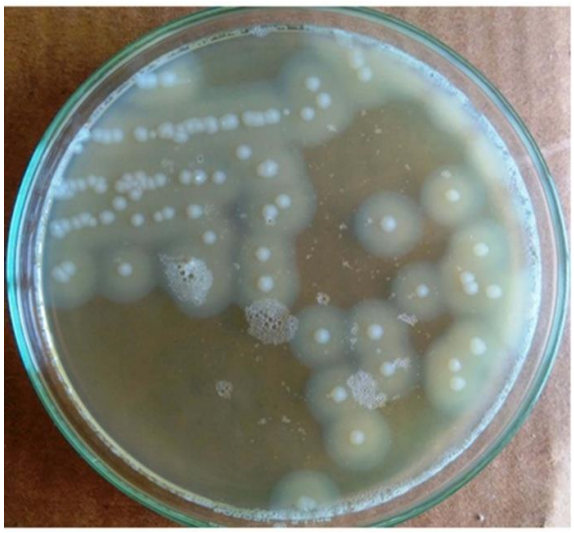
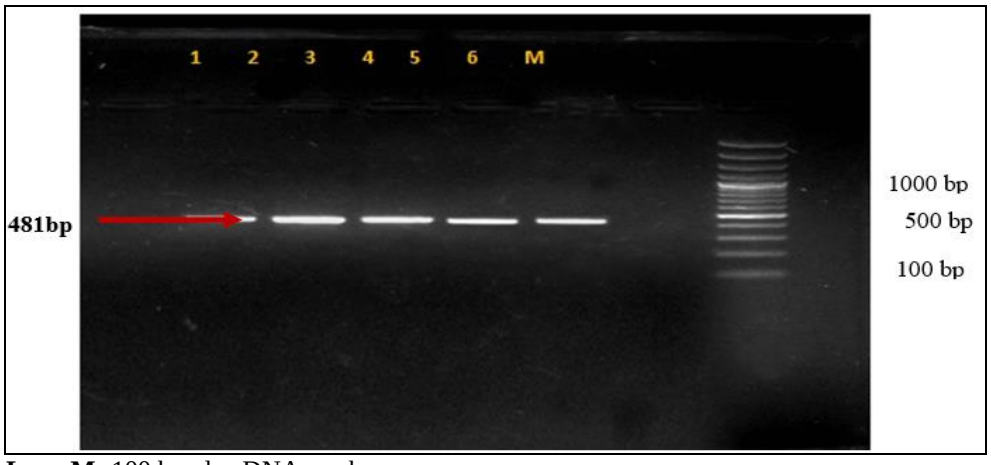
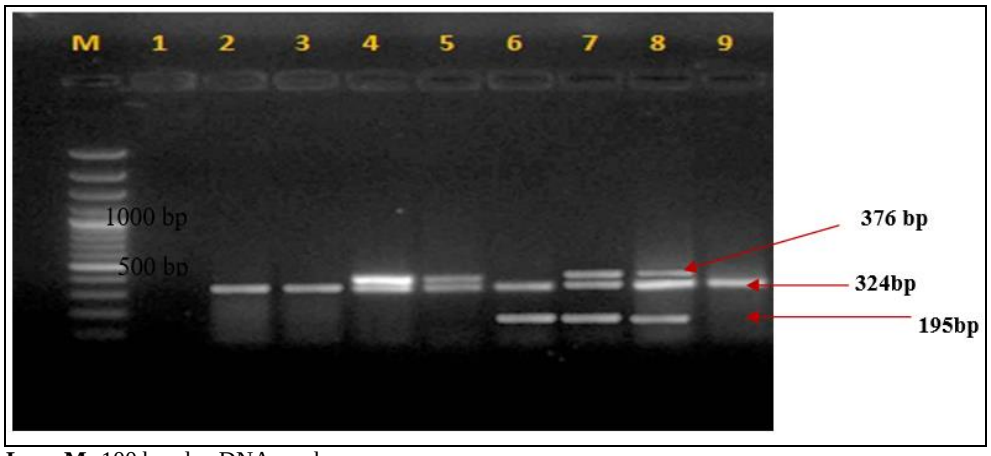


Fig 3: Lecithinase activity of *Clostridium perfringens* on egg yolk agar



Lane M: 100 bp plus DNA marker.
Lane 1-4: 481bp product corresponding to 16S rRNA gene
Lane 5: Positive control (ATCC 13124)
Lane 6: Negative control.

Fig 4: Agarose gel electrophoresis showing species specific amplified product of 16S rRNA gene of *Clostridium perfringens*



Lane M: 100 bp plus DNA marker.
Lane 1: Negative control
Lane 2: Positive control(ATCC 13124)
Lane 3: *C. perfringens* Type A with *cpa* (324 bp) gene amplification
Lane 4-5: *C. perfringens* Type D with *cpa* (324 bp), *cpε*(376bp) gene amplification
Lane 6: *C. perfringens* Type C with *cpa* (324 bp), *cpb* (195bp)gene amplification
Lane 7-8: *C. perfringens* Type B with *cpa* (324 bp), *cpb* (195bp), *cpε*(376bp) gene amplification
Lane 9: *C. perfringens* Type A with *cpa* (324 bp) gene amplification

Fig 5: Agarose gel electrophoresis of multiplex PCR amplicons of different toxinotypes and virulence genes of *Clostridium perfringens*

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