Research Article

Typing of *Clostridium Perfringens* Isolated from Broiler Chickens Using Multiplex PCR

Neda Shahdadnejad[1], Mohammadreza Mohammadabadi[1*], Mehrdad Shamsadini[2]

1. College of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran
2. Anaerobic Bacterial Vaccines Research & Production and Molecular Microbiology Department, Kerman Branch, Razi Vaccine & Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Kerman, Iran

Abstract

Clostridium perfringens (*C. perfringens*) is related to gram-positive and anaerobic bacteria. This rod-shaped bacterium produces spores and can cause a wide spectrum of diseases in humans and animals. Every of five different bacterium types can lead to different diseases. Most of distinct bacterial recognition methods are not completely effective and practical in all condition. The objective of this study was to isolate and identify different species of clostridium from the intestinal tract of broilers in Kerman province; southeastern of Iran using multiplex PCR. For doing this research, the intestinal tract samples were taken randomly from 122 broilers chickens. Samples were purveyed and cultured and then morphology of created colonies was checked and gram stain examination was performed. Genus identification of these bacteria was performed biochemical tests. Then DNA extraction from isolated bacteria was carried out and then multiplex PCR with specific primers was performed to classify toxin. Toxin types and bacterial strains were identified according to synthesized PCR fragments length. The only type of bacteria that isolated from intestinal tract of broilers was type A of *C. perfringens*. Our results indicated that *C. perfringens* type A is the most common type in intestinal tract of studied broiler population.

Key words: Broiler; *Clostridium perfringens*; Multiplex PCR; Iran

Introduction

C. perfringens, is one of the most widely distributed pathogenic bacteria in the environment, which is an anaerobic, gram-positive and spore-forming bacillus (1). This bacterium is the most rapidly growing foodborne pathogen and is found in soil, water, air, food, and intestinal tract of humans and animals (2). Probably, this microorganism is one of the most widespread pathogenic bacteria and it is undeniably the most important cause of clostridial disease in humans and animals. On the other hand, domestic animals are known to be sources of human food poisoning. In order to reduce or eliminate this risk, strategies must be developed to diagnose and prevent infected animals from entering the food chain (3). However, *C. perfringens* can produce up to 15 toxins in various combinations, responsible for the

* Mohammadreza Mohammadabadi, PhD
Professor, College of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran
Email: mrm@uk.ac.ir
Submission Date: 23 Agu. 2016 • Acceptance Date: 25 Oct. 2016

pathogenesis of the disease. *C. perfringens* is also the causative agent of clostridiosis, one of the most important diseases in broiler and turkey flocks. Due to the withdrawal of growth-promoting antibiotics and ionophorous anticoccidials in the European Union, *C. perfringens*-associated necrotic enteritis and subclinical diseases have become more serious threats to poultry health (4). *C. perfringens* does not invade healthy cells, but produces various toxins and enzymes that are responsible for the associated lesions and symptoms. The toxins produced depend on the *C. perfringens* strain involved, and each type of toxin induces a specific syndrome, viz, alpha (α), beta (β), epsilon (ε) and iota (ι) comprising the four main *C. perfringens* toxins which create the classifying foundation of this bacterium to five various kinds (5).

Therefore, the correct identification of *C. perfringens* pathovars is critical for epidemiological studies and for the development of effective preventative measures, including vaccination. Outbreaks of necrotic enteritis, caused by *C. perfringens*, have been frequently reported in chickens in all countries including Iran. However, identifying various kinds of *C. perfringens* using biochemical experiments is impossible (6). The newest applied distinguishing technology for infectious diseases is polymerase chain reaction (PCR). In comparison with classical methods, PCR is faster and more valid (7-10). Furthermore, PCR permits to recognize bacterium from clinical samples directly and rapidly (5). Genotyping, which is based on a more stable marker, DNA, is not dependent on gene expression. Another advantage of genotyping methods is that the discriminatory power of DNA-based methods is generally superior to that of phenotypic methods. The ability to distinguish between genomes is important to several disciplines of microbiological research, for example in studies on population genetics and microbial epidemiology (11). Of great importance when choosing a method for genotyping are the typing ability, reproducibility, discriminatory power and also the ease and cost of performing the analysis.

Several PCR methods have since been established for typing isolates of *C. perfringens*. Zandi et al (12) typed toxigenic isolates of *C. perfringens* by multiplex PCR in Ostrich and demonstrated that *C. perfringens* isolated types were divided as 100% type A and showed that PCR is the reliable technique for detection of *C. perfringens* isolated types. Afshari et al. (13) typed *C. perfringens* in broilers meat collected from retail meat shops in Mashhad city of Iran using multiplex PCR and demonstrated that *C. perfringens* type C is the most popular kind in broiler chicken carcasses.

Although different chickens in Iran have been studied by PCR technique to determine genetic diversity and genotype them for different loci (14-16), but until now researchers have not used PCR for typing of *C. perfringens* in intestinal tract of broilers chickens in Iran. Furthermore, as PCR typing of *C. perfringens* is important for epidemiologic surveys, the aim of this study was to determine the incidence and toxin typing of *C. perfringens* in intestinal tract of broilers collected from Kerman province in south east of Iran.

Materials and Methods

In this research, intestinal tract samples were taken from 122 broiler chickens, breed in ten aviculture in Kerman province, south-east of Iran. The sterile plastic bags were used for collecting and transferring samples to the laboratory through 1 to 2 hours. Dilution of samples was performed in PBS (1:10). For removing the non-spore-forming bacteria, the bath temperature was retained at 80°C through ten minutes. Then they were cultivated on 5% sheep blood agar and anaerobically incubated using Anoxomat® (Mart Microbiology, Netherlands) at 37°C for 48 hours. The identification of suspected colonies based on characteristic colony morphology, gram staining and biochemical tests was carried out as described earlier (6). For biochemical recognition, gelatin and lecithin hydrolyses; and catalase, lipase and motility tests were performed. Furthermore, identification of bacterial genus was performed (6) using indole production, litmus milk reaction and carbohydrate fermentation (sucrose, glucose, lactose and maltose), as detailed in Table 1.

DNA extraction was performed from isolated strains of *C. perfringens* that cultured on blood agar (17). Two or three from these colonies were suspended in 350 µL of STET buffer (100 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 5% Triton® X-100) in 1.5-mL microtubes. Amount of 25 µL from lysozyme (10 mg/mL) was subjoined to value of every solution and then was mixed. Afterwards, these microtubes were put in a boiling water bath through 40 seconds. Centrifugation of the bacterial lysate was performed at 13,000 rpm through 15 minutes at room temperature in a Microfuge® refrigerated microcentrifuge (Beckman Coulter, USA). Subsequently, the supernatant was shed into a new microcentrifuge tube and was added 40 µL of 2.5 M sodium acetate (pH

Table 1: Biochemical tests for identification some clostridia species for intestinal tract samples of broiler chickens in Kerman province

Biochemical tests	Egg yolk agar			Indole produced	Carbohydrate fermentation				Motility	Milk reaction
Clostridial species	Catalase test	Lecithinase produced	Lipase produced	Gelatin	Glucose	Lactose	Sucrose	Maltose		
C. perfringens	–	+	–	+	–	+	+	+	+	–	dc
C. baratii	–	+	–	–	–	+	+w	+	+w	–	c
C. absonum	–	+	–	+	–	+	+	+	+	±	c
C. bifermentans	–	+	–	+	+	+	–	–	–w	+	d
C. sporogenes	–	–	+	+	–	+	–	–	–w	±	d
C. leptum	–	–	–	–	–	–	±	±	+	–	–
C. rantibutyricum	–	–	+	+	–	+	+	+	+	+	c
C. orosphaeroides	–	–	–	–	–	–	–	–	–	–	–
C. symbiosum	–	–	–	–	–	+	±	–	–	±	–c
C. scatologenes	–	–	–	–	±	+	–	–	–	+	–
C. ramosum	–	–	–	–	–	+	+	+	+	–	c
C. sordellii	–	+	–	+	+	+	–	–	+w	±	d

*c: curd; d: digestion; dc: first digestion then curd; w: weak

5.2) and 420 µL of isopropanol in order to precipitate nucleic acids. These precipitated nucleic acids were centrifuged again at 13,000 rpm through ten minutes at 4°C. Then, the supernatant was eliminated and the pellet of nucleic acid was washed with 1 mL of 70% ethanol at 4°C. At the end, the compressed mass of nucleic acids was dried and treated in 50 µL of TE buffer containing RNase. Four specific primer sets (18) regarding to every toxin of *C. perfringens* were appraised for their diagnosing ability in *C. perfringens* and their discriminating ability between *C. perfringens* and other clostridia species (Table 2). The PCR was carried out in a Thermal Cycler® (Bio-Rad, USA) in a total reaction volume of 50 µL including: 5 µL of 10x PCR buffer (10 mM Tris-HCL, pH 9.0, 50 mM KCl), 2 µL of 50 mM MgCl2, 10 mM dNTP, 5 U of Taq DNA polymerase (0.1 U/µL), 10 pM/µL of each primer and 5 µL of template DNA (50 ng/µL). Amplification reaction was performed within 35 cycles subsequent a preliminary denaturation step at 95°C through ten minutes. Every cycle included denaturation at 94°C through 45 seconds, annealing at 55°C through 30 seconds, and extension at 72°C through 90 seconds. The ultimate synthesis step arisen at 72°C through ten minutes. After ending PCR, 10 µL of PCR products was electrophoresed on a 1.5% agarose gel and stained with DNA safe satin. Visualization of the amplified fragments were performed under UV light.

Results

In this research, 122 intestinal contents were analyzed and well-grounded. Results of tests showed that 77.87 % of samples (95 samples) were positive for clostridium types, indicating the sensibility of poultry towards clostridial diseases. Also, *C. perfringens* was found more widespread and popular type of clostridia genus that was isolated and contained 52.63 % of the total clostridia isolates, that included *C. perfringens* (50 samples), C. clostridioforme (14 samples), C. leptum (13 samples), C. baratii (11 samples), C. novyi (4 samples), C. subtermins (1 sample), C. celatum (1 sample), and C. chauvoeri (1 sample).

Identification of the extracted DNA from all studied bacterial strains was performed using biochemical tests and stand-

Table 2: Primers employed in multiplex PCR for intestinal tract samples of broiler chickens in Kerman province

Toxin	Gene	Sequence 5'-3'	Primer position (bp)	Amplicon (bp)
α	*plc (cpa)*	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	663-968	324
β	*cpb*	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	871-1045	196
ε	*etx*	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	267-862	655
ι	*iap*	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	1739-2161	446

ard strains. Quality confirmation of the extracted DNA was carried out on 1% agarose gel. Performing the PCR with four sets of specific primers was confirmed, and produced fragments of α, β, ε and ι encoding genes of *C. perfringens*. Amplification of complementary genes and syntheses of the fragments disclosed a specified toxin gene. Hence, various types were recognized in terms of their toxins. Using single PCR, the fragment of 324 bp belonging to the α toxin gene, as seen in all types, was synthesized and identified. But, the 196 bp fragment belonging to the β toxin encoding gene was observed only in types B and C. The 655 bp fragment was synthesized as the ε toxin encoding gene and was seen in type D. The fragment of 446 bp was from the ι toxin encoding gene and was observed only in type E (Figure 1). Figure 2 shows positive and negative controls of PCR. All of the 50 suspected isolates grown on selective agar were PCR positive for the α toxin gene of *C. perfringens*.. PCR products for the α toxin gene (324 bp) of *C. perfringens* are shown in Figure 3. All 50 diagnosed *C. perfringens* types identified by the biochemical tests were type A.

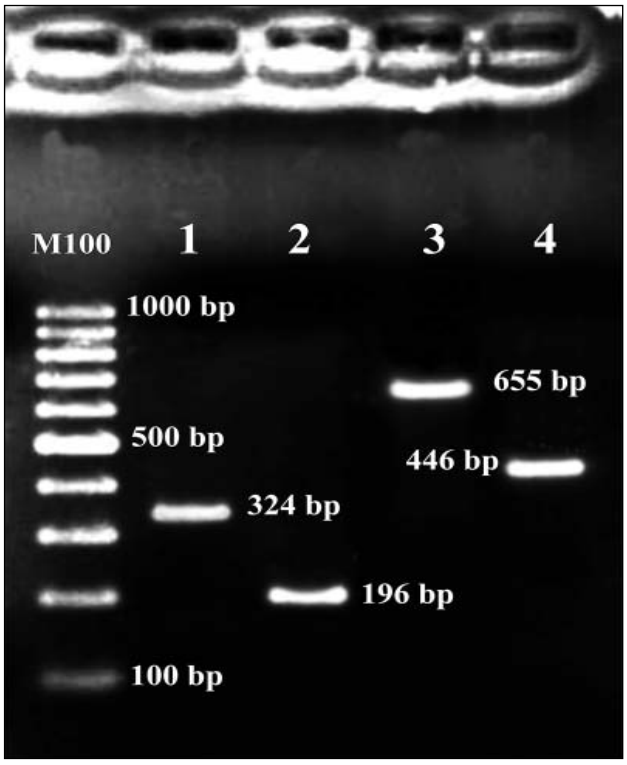

Figure 1: Detection of *C. perfringens* toxin genes amplified by single PCR. M: marker (DNA ladder, 100 bp); lane 1: α toxin encoding gene; lane 2: β toxin encoding gene; lane 3: ε toxin encoding gene; lane 4: ι toxin encoding gene

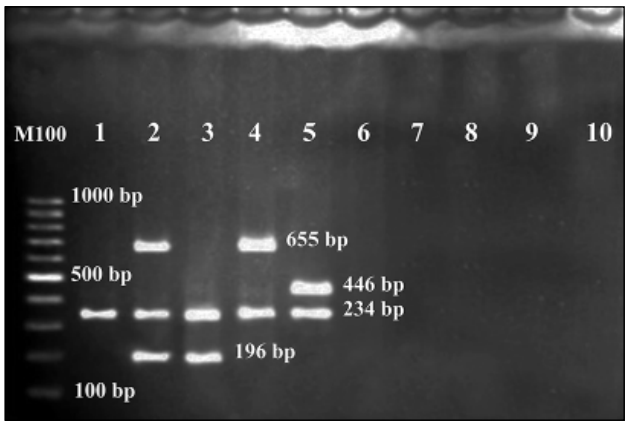

Figure 2: Standard strains, positive and negative controls. M: marker (DNA ladder, 100 bp); lanes 1 to 5: positive controls, standard strain of *C. perfringens* types A, B, C, D and E (Lane 1: cpa; Lane 2: *cpa*, *cpb* and *etx*; lane 3: *cpa* and *cpb*; Lane 4: *cpa* and *etx*; Lane 5: *cpa* and *iap*); lanes 6 to 10: negative controls, *C. septicum*, *C. sordellii*, *C. sporogenes*, *C. leptum* and *C. ramosum*

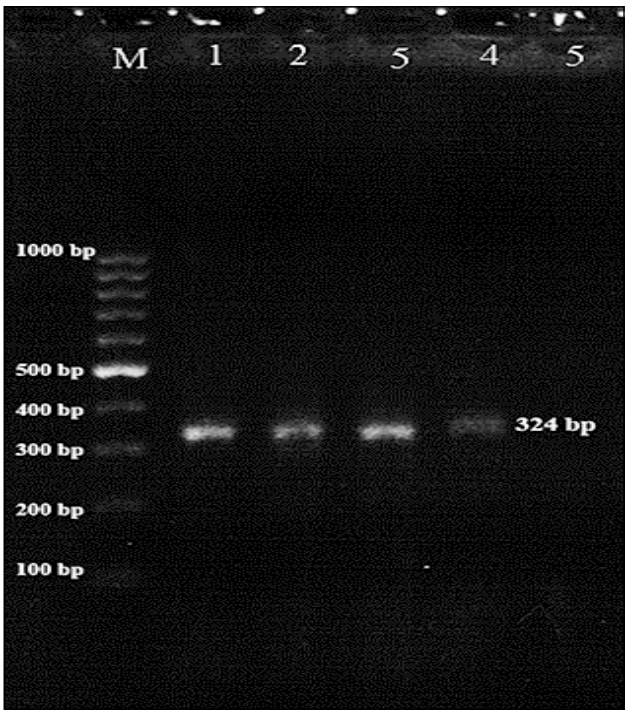

Figure 3: Agarose gel electrophoresis of PCR products obtained from *C. perfringens* isolated from a Kermani intestinal tract of broiler chicken. M: marker (DNA ladder, 100 bp); lanes 1-4: *C. perfringens* type A isolates; lane 5: negative control

Discussion

C. perfringens is a normal flora of the intestine that is usually presented in low numbers. These bacteria produce little toxin and under normal conditions are removed by normal gut motion, or are inactivated using circulating antibodies (1,19). When a sudden alteration is created in the diet of chickens by overeating seeds, bacteria will able to reproduce quickly. And when food moves slowly in the intestine, the organism reproduces and the rate of toxin elimination (neutralization) becomes slower than the production of the toxin, thereby toxemia occurs (19). Hence, inoculation against *C. perfringens* toxins is a very important step to prevent losses because of the enterotoxaemia and keeping down the intensification of diseases (1). Toxin typing of *C. perfringens* is important since particular toxin types are associated with specific enteric diseases in animals. One of the most important diagnosing procedures of *C. perfringens* is the biological methods; but, they pose many difficulties. Some of the disadvantages are the lengthy test (taking 24 to 72 hours) and non-specific deaths due to wrong detection. Furthermore, some *C. perfringens* strains cannot produce toxins in measurable amounts under laboratory conditions, which makes a barrier for typing by classic procedures (20). Two basic and essential specifications of an effective and applied method are sensitivity and specificity that PCR shows these two characteristics. Main benefit of PCR is its high speed, so that bacterium identification and type determination does less than four hours. Thus, identification of the toxicogenic strain in a sample, by using PCR technique, can be done before toxin producing. The PCR technique is a strong gadget for finding and diagnosing minimal numbers of microorganisms. A multiplex PCR, which can detect all *C. perfringens* major toxin genes, has been developed. All of the isolates from intestinal tract of broiler chickens in Kerman province were confirmed as *C. perfringens* type A through the detection of cpa gene using multiplex PCR. It is not surprising that in this study all *C. perfringens* isolates were found to be positive according to multiplex PCR results and that all of them were determined as type A. It can be explained by: (i) the fact that cpa toxin genes are common genes for all *C. perfringens* types, (ii) it is common among *C. perfringens* types worldwide, and (iii) *C. perfringens* type A is dominant in almost all researches concerning poultry (21, 22).

In the current study, only *C. perfringens* type A was isolated from broiler chicken samples in Kerman province, hence, it is recommended that inoculation versus enterotoxemia in this province must cater enough inviolability, particularly against *C. perfringens* type A. Moreover, enterotoxemia in poultry is asymptomatic; thus, the molecular primers employed in this research were excellently specific for detection of *C. perfringens* encoding toxin gene. As figures illustrate, the employed primers in this research do not recognize other strains. Moreover, PCR displayed to be very sensitive as the reaction was done prosperously with a low value of DNA (less than 1 μL). Molecular procedures generally furnish a novel intelligence into bacterial assortment. These methods pro-

pose necessary genetic information about the concerning organism, which includes one of the most applied and useful perspectives of PCR. Each single species or strain can be followed between various species or types of organisms using specific PCR primers (23). In comparison with the procedures needing large amounts of samples, PCR is more secure for investigators since sample preparation for PCR begins with cell lysis and DNA purification. It is inessential to declare that the cell cannot survive this phase and tines its infective character (23).

Conclusion

In this research, *C. perfringens* isolates were successfully typed using the multiplex PCR procedure. The multiplex PCR protocol used here caters a beneficial and valid gadget for *C. perfringens* genotyping in routine veterinary diagnostics. Insomuch only *C. perfringens* type A was isolated from broiler chicken samples in Kerman province, thus type A being the most frequentative in Kerman, and therefore it is recommended that inoculation against enterotoxemia caused particularly by *C. perfringens* type A should systematically be carried out.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors wish to thank Razi Vaccine and Serum Research Institute of Kerman, Iran, for their help.

References

1. Ahsani MR, Bafti MS, Esmailizadeh AK, Mohammadabadi MR. Genotyping of isolates of Clostridium perfringens from vaccinated and u nvaccinated sheep. Small Rumin Res2011;95(1):65-69.
2. Songer JG, Meer RR. Genotyping of Clostridium perfringens by polymerase chain reaction is a useful adjunct to diagnosis of clostridial enteric disease in animals. Anaerobe 1996;2(4):197-203.
3. Piatti RM, Ikuno AA, Baldassi L. Detection of bovine Clostridium perfringens by polymerase chain reaction. J Venom Anim Toxins Incl Trop Dis 2004;10(2):154-160.
4. Johansson A. Clostridium perfringens: the causal agent of necrotic enteritis in poultry.Ph.D. Thesis. Swedish University of Agricultural Sciences. ISSN 1652-6880, ISBN 91-576-7083-8. 2006.
5. Ahsani MR, Mohammadabadi MR, Shamsaddini MB. Clostridium perfringens isolate typing by multiplex PCR. b J Venom Anim Toxins Incl Trop Dis2010;16(4):573-78.
6. MacFaddin JF. Biochemical tests for identification of medical bacteria. Baltimore: Lippincott Williams & Wilkins 2000:1-450.
7. Miyashiro S, Nassar AFC, Fava C, Cabral AD, Silva M. Del Clostridium perfringens types A and D associated with enterotoxemia in an 18-month-old goat. J Venom Anim Toxins Incl Trop Dis2007;13(4):885-93.
8. Mohammadabadi MR, Soflaei M, Mostafavi H, Honarmand M. Using PCR for early diagnosis of bovine leukemia virus infection in some native cattle. Genet Mol Res2011;10(4):2658-63.
9. Ahsani MR, Abadi MR, Shamsaddini Bafti M, Ezatkhah M, Hasani M, Esmailzadeh AK, Hasani Derakhshan M. Application of triplex PCR technique in identification of clostridium perfringens b, c and d types. Iran J Anim Sci Res2010;2(2):185-190 (In Farsi).
10. Mohammadabadi MR, Shaikhaev GO, Sulimova GE, Rahman O, Mozafari MR. Detection of bovine leukemia virus proviral DNA in Yaroslavsl, Mongolian and black pied cattle by PCR. Cell Mol Biol Lett2004;9(4A):766-68.
11. Baums CG, Schotte U, Amtsberg G, Goethe R. Diagnostic multiplex PCR for toxin genotyping of Clostridium perfringens isolates. Vet Microbiol 2004;100(1-2):11-6.
12. Zandi E, Mohammadabadi MR, Ezzatkhah M, Esmailizadeh AK. Multiplex PCR. Typing of Toxigenic Isolates of Clostridium Perfringens by in Ostrich. Iran J Appl Anim Sci 2014;4(4):509-514.
13. Afshari A, Jamshidi A, Razmyar J, Rad M. Genotyping of Clostridium perfringens isolated from broiler meat in northeastern of Iran. Vet Res Forum 2015;6(4):279-284.
14. Mohammadifar A, Imani SA, Mohammadabadi MR, Soflaei M. Faghih. The effect of TGFb3 gene on phenotypic and breeding values of body weight traits in Fars native fowls. J Agri Biotech2013;5(4):125-36 (In Farsi).
15. Mohammadabadi MR, Nikbakhti M, Mirzaee HR, Shandi MA, Saghi DA, Romanov MN. Genetic variability in three native Iranian chicken populations of the Khorasan province based on microsatellite markers. Russ J Genet2010;46(4):505-509.
16. Moazeni SM, Mohammadabadi MR, Sadeghi M, Moradi Shahrbabak H, Esmailizadeh AK, Bordbar F. Association between polymorphisms of growth and reproductive traits in mazandaran indigenous chicken. Open Journal of Animal Sciences 2016;6(1):1-8.
17. Sambrook J, Russell DW. Molecular Cloning:ALaboratory Manual. third ed. Cold Spring Harbor Laboratory Press 2002;18-96.
18. van Asten AJAM, van der Wiel CW, Nikolaou G, Houwers DJ, Grone A. A multiplex PCR for toxin typing of Clostridium perfringens. Vet Microbiol2009;136(1):411-412.
19. Gokce HI, Gokce G, Cihan M. Alterations in coagulation profiles and biochemical and haematological parameters in cattle with traumatic reticuloperitonitis. Vet Res Commun2007;31(5):529-537.
20. Kalender H, Ertas HB, Cetinkaya B, Muz A, Arslan N, Kilic A. Typing of isolates of Clostridium perfringens from healthy and diseased sheep by multiplex PCR. Vet Med – Czech 2005;50(10):439-442.
21. Guran HS, Oksuztepe G. Detection and typing of Clostridium perfringens from retail chicken meat parts. Lett Appl Microbiol 2013;57(1):77-82.
22. Nowell VJ, Poppe C, Parreira VR, Jiang Y-F, Reid-Smith R, Prescott JF. Clostridium perfringens in retail chicken. Anaerobe 2010;16(3):314-5.
23. Bartlett JMS, Stirling D. eds.PCR Protocols (Methods in Molecular Biology). 2nd ed. Humana Press 2003; 3-115