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THE DIAGNOSIS AND EFFECT OF BREAST TUMORS TREATMENT IN DOGS

Narziev Baxtiyor and Yulchyiev Jasurbek

Address(es): Yulchyiev Jasurbek,
Samarkand Agricultural Institute, Veterinary Medicine Faculty, Mirzo Ulugbek 77, Samarkand, Uzbekistan, +998662343320.

*Corresponding author: jasur_vet@mail.ru

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ABSTRACT

This study reports the spread of tumors of mammary gland in dogs. Changes in blood immunological and morphological parameters of affected dogs are presented. Results of immunotherapy used for activation of immune system after surgery showed various level of stimulation. Application of levamisole increase the percentage of T- and B-lymphocytes as well as the ratio of normalized subpopulations of T lymphocytes (T-helper and T-suppressor cells) indicating potential effect of levamisole. Application of BCG improved performance of T- and B-lymphocytes, but in comparison with the effect of levamisole some imbalance between subpopulations of T-helper and T-suppressor cells was detected. In the control group (simple tumor removal) different results were found indicating immunosuppressive effect of tumors.

Keywords: tumor, immunity, lymphocytes, therapy

INTRODUCTION

In recent years a significant development occurs in the study of cancer diseases in animals and especially in dogs. The reasons that lead to the appearance of tumors in dogs are (1) those associated with the occurrence of tumors in farm animals, often worn by the character of widespread, requiring the adoption of appropriate measures, based on scientific research, and (2) increased interest in problems comparing tumors in humans and domestic animals, as the latter the "model" in the study of human tumors and significant role in the expansion of knowledge about the nature of tumor growth providing appropriate care in the treatment of tumor development **(Gardner *et al.*, 2015).** It should be noted that according to the scientific and experimental studies, dogs have the same sensitivity to carcinogens as gas pollution, ionizing radiation, solar radiation etc. Furthermore, tumors in dogs develop after the start of exposure to carcinogens much faster than in man. With this in mind, as well as the fact that dogs live in the same environment, and often eat the same food, drink the same water as the man a study of tumors for early detection of possible human carcinogen environmental factors is quite urgent. Identifying similarities in development of certain tumors in dogs and humans can be considered as natural models and can be used for study of the development of human tumor, their diagnosis and the development of new treatments. Finally methods of diagnosis and treatment of tumors in humans may be used in relation to tumors in dogs **(Regan *et al.*, 2015; Weisse 2015)**.

The most common types of cancer in dogs are tumors of the breast. Mammary gland tumors in dogs range from 25 to 41% of all neoplasia (**Horn and Horn 1971; Terekhov, 1983; Melief 2000; Artamonova, 2002; Chalyiuv *et al.*, 2002; Chagpar *et al.*, 2004; Haberkorn *et al.*, 2013**). According to several authors (**Terekhov 1983; Artamonova 2002; Gonzalez-Angulo *et al.*, 2007**) mammary tumors in dogs has an impact on the weight of the body, including the immunity. The problem of immunological reactivity in tumor growth remains one of the most important scientific problems of modern medicine and veterinary medicine.

The promise of pharmaceutical biotechnology and its applications to health care is now being realized. Therapeutic agents produced by the tools of biotechnology are now available. Recombinant proteins, monoclonal antibodies, gene therapy, and vaccines can provide effective treatments and cures for diseases we could only mitigate in the past. The availability of these drugs presents several interesting challenges and opportunities to pharmacists. The expense, complexity, and unique properties of biotechnology agents will continue to alter not only the knowledge base of pharmacy but also the way pharmacists perform their professional responsibilities (**Piascik, 1998**).

Aim of study was to analyze the effect of levamisole and BCG on selected immunological and hematological parameters in dogs with cancer pathologies.

MATERIAL AND METHODS

In this study dogs with tumors of the breast, received at the Department of Anatomy, Physiology, Surgery and Pharmacology of Animals, Samarkand Agricultural Institute were analyzed. From 150 dogs examined breast tumor of various sizes was detected in 45 cases. Dogs were divided into 3 groups: 2 experimental and control.

All dogs were subjected to surgical intervention to remove breast cancer. In the first experimental group (E1) dogs received levamisole (intramuscular 75 mg two injections a week); in the second experimental group (E2) Bacillus Calmette–Guerin (BCG, subcutaneously 0.5 mg once per week). The third group served as a control in which only the surgical removal of the tumor was realized.

For general anesthesia i.v. 2.5% solution of chlorpromazine (1 ml per 10 kg body weight), 5% ketamine (1 ml), and in some cases for complete relaxation intramuscular injection of 2% xyla (0.15 ml per 10 kg body weight) was used. For local anesthesia 0.5% novocain solution was used, injected between the skin and the capsule of the tumor.

After each injection the blood was analyzed for hemoglobin, erythrocytes, leukocytes and leukocyte formula by conventional methods, T- and B-lymphocytes were determined by plaque assay. Diagnosis was based on clinical and histological studies. Clinical examination included an assessment of the primary tumor sites, regional lymph nodes, and the general condition of the animal.

RESULTS AND DISCUSSION

For removing the breast tumor straight-line or spindle-shaped incision was used, depending on the size of the tumor. After making the skin incision blunt was capable for skin separation from the tumor capsule. It was necessary to stop the bleeding during the operation mechanically. After removal of the breast tumor performed skin grafting was done and interrupted skin sutures were applied. In cases of metastasis in the regional lymph nodes they were also removed. Sutures were removed on day 7-10.

In the first group (E1) with levamisole application the level of T-lymphocytes before the therapy was 38.3±2.6% after the applications 53.3±0.3% of white blood cells (277.4±0.6 vs. 1271.5±0.8 per µl). The level of B-lymphocytes was before therapy 4.3±0.3% and after the therapy reached 15.7±1.0% (31.6±0.8 vs. 374.2±1.5 per µl). For subpopulation of T-lymphocytes the relative occurrence of T-helper cells was 26.6±1.6% vs. 36.3±1.6% and T-suppressors 7.6±0.1% vs. 8.0±0.6% after therapy (Figure 1).

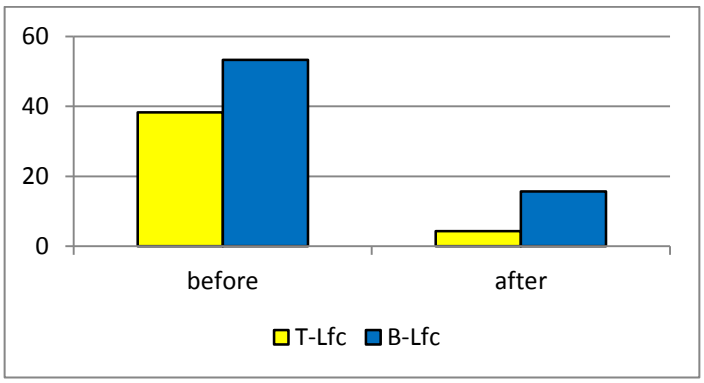

Figure 1 Percentage (%) of T- and B-lymphocyte of white blood cells in the group E1 – levamisole application

In the second group (E2) the BCG immunotherapy was conducted once a week. The relative amount of T-lymphocytes prior to application was 38.6±1.3% and after application 53.6±3.0% (552.9±2.9 vs. 2702.0±7.5 per µl). The number of B-lymphocytes increased from 5.6±1.0% to 18.0±0.6% (83.0±4.0 vs. 915.3±2.7 per µl). In the study of subpopulations of T-lymphocytes the level of T-helper cells increased from 27.0±0.7% to 38.6±2.3% and T-suppressor cells from 8.3±0.6% to 17.6±0.9%. BCG stimulated the immune system with clear increase of the levels of T-lymphocytes and their subpopulations (Figure 2).

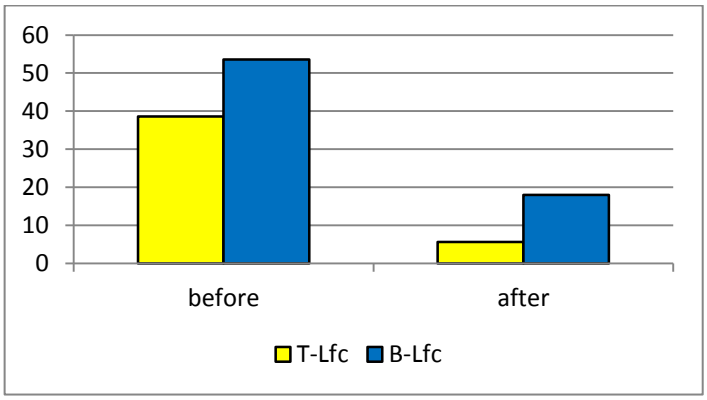

Figure 2 Percentage (%) of T- and B-lymphocyte of white blood cells in the group E2 – BCG application

In the control group (surgical intervention) results were different (Figure 3). A decrease of the relative level of T-lymphocytes was found – 40.6±2.6% vs. 34.0±1.3% (376.7±0.7 vs. 233.0±1.0 per µl). The percentage of B-lymphocytes was 9.0±2.0 vs. 8.6±2.3% (83.1±2.0 vs. 58.3±1.6 per µl). In the subpopulation of lymphocytes T-helper cells decreased from 26.3±0.3% to 23.0±3.0% with simultaneous increase of T-suppressor from 12.6±0.8% to 18.3±2.6%.

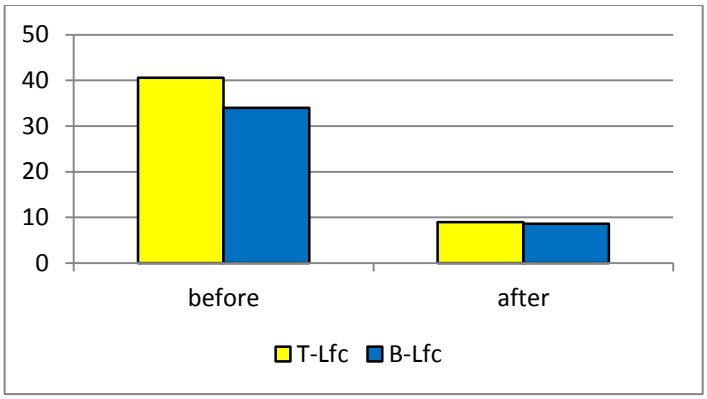

Figure 3 Percentage (%) of T- and B-lymphocyte of white blood cells in the control group – only the surgical removal of the tumor

Generally cancer suppresses the immune system. In the investigation of immunotherapy to blood in dogs a decrease of lymphocyte to 6-8% was observed. After removal of tumors in dogs the immune system was stimulated in groups with BCG and levamisole application.

Cytological or histological examination of tissue is usually used for tumor identification. Instrumental methods as X-ray or ultrasound are used to find distant nodal or hematogenous metastases. Clinical signs of breast cancer are paraneoplastical syndromes (**Piane *et al.*, 2014**), which usually do not develop immediately and strongly time lag in the development of the visible changes of the breast configuration. Deformation can form single or multiple units of various sizes, texture, density, with or without cysts affecting one or more packets of the breast, often painful, but sometimes accompanied by pain and signs of inflammation with broken or intact skin over the deformation (**Lim *et al.*, 2015**).

Morphological (cytological and histological) diagnosis of tumors of the mammary glands is required for the following tasks - differential diagnosis of breast tumors from benign diseases, identification of breast cancer to develop tactics for further diagnostic and therapeutic measures, determination of the histological characteristics of the tumor (type, germination of the capsule, the degree of malignancy) for the development of operational tactics after the treatment and control of metastasis and prognosis of outcomes, morphological study of regional lymph nodes for detection of nodal metastases as well as cytology performed to detect cancer cells by aspiration or biopsy (**Munson and Moresco, 2007; Klopfleisch *et al.*, 2011**).

Immuno–biological studies of the interaction of organisms with tumor cells and tissues show that the determining factor in the prevention of the emergence and development in the effective elimination of tumor cells is the stimulation of both specific and non-specific defense systems of the body and immune system as a whole **(Eifel *et al.* 2001).**

It is also known that the most important are the problems of cancer therapy. It was found that many drugs and methods for treating tumors give quite good results, even in the treatment of experimental tumors were unsuitable or quite effective in the treatment of spontaneous animal and human tumors. That is why the use of affected animals with spontaneously occurring tumors seems more promising to clarify many issues genesis blastoma process research to develop effective treatments for tumors of humans and animals **(Terekhov, 1983; Melief 2000; Chalyiuv *et al.* 2002)**.

The most effective treatment is the biophysical and/or biochemical processes against pathological processes that lead to disease. However, the study of the phenomenon known as “the body's resistance” is the first step. Significant theoretical advance in oncology was the discovery that cancer is caused by a specific immune response in the body, in which it appears (**Brtitta *et al.*, 2005; Haberkorn *et al.*, 2013).**

Many growing tumors suppress the body immunity. Immunosuppressive effect is particularly evident in the advanced stage of cancer (also tumors of small size). There is little doubt that the immune response plays an important role in the growth and development of tumors **(Melief *et al.*, 2000; Luzhna *et al.*, 2013; Artamonova *et al.*, 2002).** According to animals in which malignancy is caused by a virus the immune system is suppressed. First, cell-mediated immunity is suppressed, directly or indirectly related to the effect of the virus on lymphoma cells. The result is a total suppression of the immune system in the process of formation and growth of tumors (**Chalyiuv *et al.*, 2002; Haberkorn *et al.*, 2013**). The actual problem of the trends in the treatment of oncological pathologies is the immunotherapy and enhancing immune reactivity (**Terekhov, 1983; Artamonova *et al.*, 2002; Haberkorn *et al.*, 2013**). Immunotherapy is a treatment of disease by active and passive immunization. The task is to find effective immunization of the patient against the tumor as described also in this study.

CONCLUSION

Cancer markedly declines the immune status of the body, reducing the rate and quantitative level of T-lymphocytes, with the predominance of suppressor activity of helper. Application of levamisole increase the percentage of T- and B-lymphocytes as well as the ratio of normalized subpopulations of T lymphocytes (T-helper and T-suppressor cells) indicating potential effect of levamisole. Application of BCG improved performance of T- and B-lymphocytes, but in comparison with the effect of levamisole some imbalance between subpopulations of T-helper and T-suppressor cells was detected. In the control group different results were found indicating immunosuppressive effect of tumors.

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TREATMENT OF ASEPTIC DISEASES OF LIMB DISTAL PART JOINTS IN UZBEK SPORT HORSES

Niyozov Hakim, Davlatov Numon, Dilmurodov Nasriddin*

Address(es):
Samarkand Agricultural Institute, Veterinary Medicine Faculty, Mirzo Ulugbek str. 77, 14103, Samarkand, Uzbekistan, +998662343320.

*Corresponding author: jasur_vet@mail.ru

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ABSTRACT

In this study a treatment was prescribed for the horses with the diagnosis of chronic aseptic synovitis and periarticular fibrosis. For all experimental groups (n=3) there was used the traditional method of treatment with only one difference, that for animals of the 1st group additionally auto–blood therapy processed with laser was used. For the animals of the second group there was injection auto–blood intramuscularly 0.5 ml.kg^{-1} of body mass of an animal and the preparation "Hondralon" in the doze of 2 ml intra-joint which strengthens the regeneration of cartilage tissue. And animals of the 3rd experimental group were treated only with traditional method.
On the base of clinical observations, morphological, biochemical and immunological indexes of blood the most effective method of treatment of joint diseases of horse limb distal part was proved- application of parenteral injection of auto-blood and specific preparation "Hondralon" together with traditional methods of treatment and the period of treatment was reduced to 5 days in average.

Keywords: Chronic synovitis, periarticular fibrosis, joints, therapy, blood analysis, treatment

INTRODUCTION

It was convincingly proved in the theories of leading classics of veterinary medicine (Pavlov and his followers) that the unity of an organism and outward environment essentially influences on all the vitally important functions of organism by the central nervous system. According to this theory all the pathological processes in the organism of animal are accompanied with continuous stimulation and overstimulation of cortex of big cerebral hemispheres of brain with the following lowering of its coordinating functions (**Rebesco and Miller 2011**). It leads to the breach of protective–adaptive processes, and later painful processes can be developed. The deep functional breaches take place in organs, tissues, and systems of an organism. In the cortex of big cerebral hemispheres of brain there are formed stable centers of overstimulation, and then inhibition, which in its turn, lead to regeneration of under cortex centers and are characterized by the formation of functional breaches. In peripheral organs these breaches are mainly connected with application of horses in sport contests and national games: kupkari, olomon poyga, kiz kuv and others (**Dolzhenko, 1977**). It is necessary to mark, that these contests are often held not on specially prepared places, but on uneven ground of foothill zones with stony soil, which leads to the breaches of the function of locomotor apparatus of horses and, especially of distal section of limbs, where the join of simple and complex joints of skeletal limbs of an animal takes place anatomically – topographically. It is necessary to indicate that these contests consists of the set of movements, which are not characteristic for a horse, that is why these animals are subjected to different traumas and, particularly to joint diseases. That is why the traumas are reasons of these diseases in pathogenesis (**Bayer and Fröhner, 1908; Shakalov, 1952; Catcoot and Smithcors, 1972; Upson, 1993; Grinaf *et al.* 1997**).
However, the analysis literature shows that at the beginning of arthrological pathology the conditions of keeping and feeding animals, for example clogging the grains with poisonous plants are of important significance (**Ibragimov and Tovmasyan, 1973; Plakhotin *et al.* 1981; Boymuradov *et al.*, 1986; Norboyev, 1990; Davlatov, 1993; Gurevitch, 2000; Livanova, 2000**). The reason of arthropathies of domestic animals can be diseases of joints with toxic of allergic origin. Besides, the sharp change for the worse of ecological region state leads to lowering of the resistance of animal organisms, which can also be the reason joint diseases. All this needs a new approach to the solution of the problems related to etiopathogenesis, modern methods of diagnostics, treatment and elaboration of preventive measures of the joint of distal part of limbs of horses, mainly used in sport purposes and for national competitions.

MATERIAL AND METHODS

Horses (n=15) belonging to OAS "Tur Orient", situated in the territory of Samarkand district of Samarkand region, participating in national games were the objects of the investigation. Animals were chosen according to the principle of pairs – analogues: 5 – 7 year old stallions of Karabair breed with the diagnosis of aseptic fibrosis and synovitis of crowning joint of distal limb part. The selected animals were distributed into 3 equal groups and kept in the stable racecourse. Every horse was kept in separate place. All experimental animals were under equal conditions of keeping and feeding according to the ration, which was made by the farm.
The horses of the first experimental group were treated with traditional method of treatment and also auto-blood (0.5 ml.kg^{-1} body mass) processed with low–frequent laser. The horses of the second group were also treated with traditional method, but the difference is, that additionally auto-blood and "Hondralon" (Chondrolonum®; http://www.rlsnet.ru/tn_index_id_6222.htm) were used. Auto-blood, processed by low-frequent laser were injected intramuscularly 0.5 ml.kg^{-1} of body mass of an animal gave an effect of stimulation, and the preparation "Hondralon", which has a power of function recovery of cartilage tissue and has an effect to stimulate additionally the joint anatomical element, was injected intra-articular (2 ml). The horses of the 3rd experimental group with the diagnosis of aseptic synovitis and periarticular fibrosis of joints were treated by massage with dioxide of mercury ointment during 12 – 15 minutes in the region of the joint. Then there was applied a compress therapy with ethyl spirit. This treatment was conducted in every 48 hours during the experiment up to full recovery.
For carrying out the set tasks the irradiation of auto-blood obtained from experimental animals was conducted. For this purpose there was used low-energetic helium-neon laser apparatus of "Shifo" type with the power of 15 mV and the length of waves of 632.8 nm. The irradiation of blood was conducted by the method elaborated by the members of the chair of Samarkand Agricultural Institute as follows: for irradiation there were necessary 2 medical bottles with 250 ml capacity, one of which is filled with stabilized 5% solution of sodium citrate in blood at the rate 1:20. For intravenous injection there was inserted a clear tube made of quarts glass into the apirogenous system, observing the hermetic ness of the device. The irradiation of blood was conducted by pouring the blood from one bottle into another, turning the ray to the quartz tube. The speed of pouring the blood were regulated by the tab, included into the system, adhering the speed of running of blood, set for laser irradiation, correspondingly 0.33 ml.sec^{-1}. For the study of the system it was necessary to insert the medical needle into both bottles for passing the air and creation the needed pressure inside the blood, colored by method of Giemsa-Romanovski. The content of hemoglobin was determined by haemometer Sali (**Kudravtsev *et al.* 1969**); the

general protein – refractometrically with the help of refractometer (IRF-22), the protein fractions by express nephelometric method of Olla–Mocord in the modification, the percent number of T–lymphocytes (E-POK) and B–lymphocytes (EAC – POK) in peripheral blood with the following summing up their absolute number and content of immunoglobulins of A, M, G class in the serum of blood by method of PHD on methodic recommendations, elaborated at AUSRIH and AUSRI.

The obtained results were processed in figures by method of student and Fisher test with summing the average – arithmetic size (m), average – arithmetic mistake (±m) the percent correlation (%) and the degree of authenticity of differences (P) with the application of computer Microsoft Excel.

RESULTS AND DISCUSSION

On the base of anamnesis, clinic, morphological, morphometric, regenerative and degenerative symptoms of the current of pathological process by general and special methods there was diagnosis of chronic synovitis and periarticular fibrosis of distal limb part of experimental horses. It was ascertained that exactly this part is the least protected with surrounding periarticular tissues. Besides, first of all outer factors from dorsal sides and namely foreign traumatic objects influence this part. For more exact diagnostics of experimental animals there was determined the placement of pathological process in the chest and pelvis limbs, which is very important for the diagnostic and prognosis point of view. Thus, on the base of thorough analysis the animals were distributed in the following way. At the experimental horses of the 1st group had the diagnosis fibrinous synovitis of embarrass joint and 3 animals had the diagnosis of periarticular fibrosis of jumping and put joint.

Three animals of the first group had diagnosis of chronic synovitis of carpal and metacarpal joints and at other heads – periarticular fibrosis of carpal joint. Data show that in the 1st experimental group in 2 cases they were located in the zone of distal joint. In the experimental horses of the 2nd group 2 cases in the carpal and one case in the metacarpal joint and in experimental animals of the third group the diagnosis of chronic synovitis in tarsal and metatarsal joint was detected. In the 3rd experimental group at 3 of animals had diagnosis of periarticular fibrosis of carpal and metacarpal joints and 2 showed chronic synovitis of distal joint. For searching for the placement of chronic synovitis there was revealed, that mainly they are placed in the joint of distal part of the limb.

It is necessary to mark that clinical symptoms of chronic synovitis in all the experimental groups were identical and characterized in the following way – at the immobility state the affected limb was kept in half–bent position in the hoof–joint, slightly leaning the grapping part of the hoof against the soil. The main weight of the body was transferred to the healthy, not injured limb.

During the animal locomotion the dependence of the degree of pathological process was observed and the lameness of slight and average degree was marked. Besides, there was marked infiltration and a slight increase of the configuration of the joint. The reaction of the joint to the outer irritation was preserved. Palpation of the joint marked the raise of the local temperature and painfulness, sensibility of the skin was slightly lowered, infiltrated and a less elastic. In the crown joint a slight deformation was found. The joint was enlarged and the diverticles were tense. The skin of the joint was elastic, mobile and sensitive.

For chronic synovitis the tense of the joint capsule with the loss of elasticity was detected and the amount of synovial liquid was reduced. At the deep palpation an increase of the amount of fibrin was marked. At the passive joint motion the pain and limitation of the joint function was found. It should be indicated, that the revealed clinical symptoms of chronic fibrous synovitis were of the same type. However, the degree of acuteness of clinical symptoms depended on the heaviness of pathological process, and also on the amount of fibrous tissue.

The examination of the location of periarticular fibrosis revealed that they are mainly located in the joints of distal limb part. The analysis of obtained data showed that the most frequently periarticular fibrosis is found in the chest limbs, and concretely in the carpal and metacarpal joints.

After treatment of affected horses by traditional method of treatment they were injected auto-blood (0.5 $ml.kg^{-1}$ body mass) which was processed with low-frequent laser. Regenerative processes after the first 24 hours after injection of auto-blood showed not significant changes in clinical indexes.

In the course of observation of the clinic status of animals in the first group in the process of treatment there were marked the following changes – during the first 48 hours after injection of auto-blood a slight increase of the body temperature, the pulse frequency pulse and breathing was observed. In animals with fibrous synovitis the local temperature raised and hyperemia was found on non-pigment parts of the skin next day after rubbing the irritating ointment and application of spirit-drying compress. The skin in the place of dorsal part of the joint was tense, hypodermic and synovial burses of the joint were well palpated. The bounds of the joint were pronounced and in tendon-joint apparatus at deeper palpation a pain was marked.

During the motion the lameness of medium degree of the leaning limb was marked. After clinical examination in the first group after 7-10 days the configuration of the joint showed no difference from the joint of opposite limb, the tension of joint capsule was lower and the state of vascularization of unpigmented parts of the joint was also reduced. In 10 days the clinical indexes in the 1-st group were in physiological range. It should be marked that the treatment of chronic synovitis with application of auto-blood processed with low-frequent laser and traditional methods of treatment for 14 days showed that the clinic-morphological and functional data of distal limb joints parts of sport horses did not differ from the same joints of healthy animals. Palpation of the joint showed marked increase of local temperature and painful reaction. On the day 5 – 6 of the experiment changes were vividly pronounced and showed the response reaction to the application of local medicinal means. On the day 10 – 12 there was a gradual rehabilitation of the elasticity of derma around the joint and diminishing of periarticular tissues. For passive joint motion a slight lameness was discovered. On the day 18 day observations showed that animals were clinically healthy.

In our experiments with treatment of chronic fibrous synovitis and periarticular fibrosis in the second experimental group with injection of auto-blood processed with low-frequent laser in the dose of 0.5 $ml.kg^{-1}$ of body mass, traditional method of treatment and intra-joint application of the “Hondrolon” for the regeneration and normalization of cartilage tissue was used. After 48 hours after the treatment marked increase of local temperature, the joint reddening and better of vascularization was found. The raise of body temperature was detected in most experimental animals. On the day 7 – 8 decrease of inflammation in periarticular tissues and compression of synovial formations were found. The full recovery of animals with diagnosis of periarticular fibrosis was observed after day 16.

The treatment of experimental animals of the 3rd group was conducted by traditional method. After 48 hours any significant differences were found. At passive and active motion the lameness of the injured limb was marked. On day 11 – 14 decreased painfulness was detected, the joint capsule and diverticules of the joint were compressed and reduced in size. At day 18 the rehabilitation was marked and the duration of the treatment of chronic synovitis lasted 18 days.

It is necessary to mark that the degree of acuteness of clinical–morphological, protective–adaptation and regenerative functions depended on the method of application of physical–chemical influences on the organism, and also the application of medicinal remedies, which were reflected on the hematological indexes.

The changes of in hematological indexes at different methods of treatment of chronic synovitis and periarticular fibrosis are listed in Table 1. In the first group the amount of erythrocytes increased and the amount of lymphocytes increased. In the second experimental group in the blood the amount of erythrocytes raised and the amount of leucocytes correspondingly increased. The change in the third experimental group on the 15th day of examination detected increase of the number of erythrocytes. Other parameters also increase, but the differences were not significant. Data show weakly the protective–adaptable and regenerative processes after application of traditional method of treatment.

Table 1 Changes of hematological indexes in experimental groups

Parameter	Before treatment	After treatment
Experimental group 1		
Erythrocytes ($10^9.\mu l^{-1}$)	5.90 ± 0,11	7.00 ± 0.10
Lymphocytes ($10^6.\mu l^{-1}$)	6.40 ± 0.26	8.30 ± 0.24
Hemoglobin ($g.l^{-1}$)	97.00 ± 3.84	130.00 ± 1.19
Lymphocytes (%)	29.00 ± 0.79	36.00 ± 0.61
Experimental group 2		
Erythrocytes ($10^9.\mu l^{-1}$)	6.30 ± 0.22	7.30 ± 0.14
Lymphocytes ($10^6.\mu l^{-1}$)	7.10 ± 0.15	9.40 ± 0.22
Hemoglobin ($g.l^{-1}$)	100.60 ± 2.56	166.60 ± 1.09
Lymphocytes (%)	30.00 ± 0.79	38.00 ± 1.69
Experimental group 3		
Erythrocytes ($10^9.\mu l^{-1}$)	6.40 ± 0.23	6.50 ± 0.14
Lymphocytes ($10^6.\mu l^{-1}$)	6.70 ± 0.14	7.20 ± 0.19
Hemoglobin ($g.l^{-1}$)	99.00 ± 1.45	102.00 ± 0.93
Lymphocytes (%)	32.00 ± 0.79	33.60 ± 1.03

In the serum of the first experimental group at the end of observation the amount of general protein increased from 59.00±0.11 $g.l^{-1}$ to 70.00±0.77 $g.l^{-1}$ and alfa globulins from 18.10±0.48% to 18.40±0.42% (Table 2). The content of gamma globulins increased from 20.40±0.80% to 25.20±1.07%. The content of albumin decreased as well as the amount of beta globulins. The examination of proteins and protein fractions of blood serum showed, that considerable changes take place in the second experimental group. The amount of general protein increased in comparison with indexes before the beginning of the experiment. Also the amount of albumins and alfa globulins decreased. At the same time there was marked increase of the amount of beta globulins and the content of gamma globulins increased from 17.90±0.83% to 23.60±1.07% in comparison with initial data. For the experimental animals of the 3-rd group before and at the end of the experiment there was marked a slight increase of general protein, the amount of albumins decreased and there was a marked increase of alfa globulins. The content of gamma globulin increased from 20.80±0.67% to 23.60±0.87% (Table 2).

Table 2 Biochemical parameters blood in experimental animals

Indexes	Before treatment	After treatment
Experimental group 1		
Proteins ($g.l^{-1}$)	59.00 ± 0.11	70.00 ± 0.77
Alpha globulins (%)	18.10 ± 0.48	18.40 ± 0.42
Gamma globulins (%)	20.40 ± 0.80	25.20 ± 1.07
Albumin (%)	41.30 ± 0.57	37.70 ± 0.95
Beta globulins (%)	20.20 ± 0.32	18.70 ± 0.22
Experimental group 2		
Proteins ($g.l^{-1}$)	61.00 ± 1:45	73.00 ± 0.79
Alpha globulins (%)	42.50 ± 1.64	37.20 ± 0.51
Gamma globulins (%)	18.90 ± 0.38	16.30 ± 0.47
Albumin (%)	20.60 ± 0.31	22.90 ± 0.71
Beta globulins (%)	17.90 ± 0.83	23.60 ± 1.07
Experimental group 3		
Proteins ($g.l^{-1}$)	62.00 ± 0.79	64.00 ± 1.36
Alpha globulins (%)	40.70 ± 0.54	37.80 ± 0.94
Gamma globulins (%)	17.90 ± 0.62	16.60 ± 0.29
Albumin (%)	20.80 ± 0.67	23.60 ± 0.87

For immune biological index the most evident changes were obtained in experimental groups 1 and 2, where auto blood processed with laser was used (Table 3). The immune biological indexes of blood of the experimental group 1 showed that the relative content of T-lymphocytes increased. Also the amount of B lymphocytes increased from 21.60±0.44% to 27.60±0.90%. The most acute changes were observed on the day 10 of the 2-nd group and were characterized by increase of the amount of relative number of T lymphocytes, absolute number of T lymphocytes, relative number of B lymphocytes and also the amount of absolute number of B lymphocytes. The examination of the blood serum of the 3-rd group showed less evident changes (Table 3).

The injection of auto-blood processed with laser together with pronounced stimulating action favorably influences on the physiological function of the connective tissues, and "Hondralon" improves the regenerative function of intra joint cartilage. It is important to mark for this method of treatment the recovery takes place without any post effects, the duration of the disease shortens and the main thing is that the expenditures for treatment of sick horses are reduced (**Frisbie *et al.*, 2015**). Arthroscopy and microscopy indicated that defects in the autologous cell group had significantly better repair tissue compared with defects in the fibrin-only and control groups. Repair tissue quality in the allogenic cell group was not superior to that in the fibrin-only group with the exception of the percentage of type-II collagen, which was greater.

Almost 15% of animals taking part in sport competitions and 22% of horses participating in kupkari games meets joint diseases. Lately in the world practice it is recommended to use methods of stimulating therapy (tissue therapy) for treatment of chronic processes of distal part of limbs. The employment of stimulating pharmacological remedies widely applied in veterinary medicine influence local and central nerve system and stimulates vitally important organs and also regulates locally the regenerative–rehabilitative processes. It is ascertained that the stimulating therapy, influencing through the central nerve system exercises the pathologic and stimulating influence. Thus, the methods of treatment are directed to the intensification of proliferative occurrence (**Gurevitch, 2000**). They improve the blood and lymph circulation and resolve the fibrin formations. This leads to intensification of fibrin–plastic processes, which contribute to dilution of fibrin formations and prevent the formation of scars in the connective tissues which leads to the breach of the function of joints. For treatment of these diseases there are widely used different methods as radiation therapy (ultra–violet, infra–red, ultra–sound radio waves with high frequency and many other. Also the electric–quantum energy is used for therapeutic purpose (**Farrelly and McEntee, 2014**). In the last years laser rays were widely used with stimulating influence. The low–frequent laser ray has a special pathogenic influence. There is also ascertain favorable influence of laser rays on blood producing organs, saturation of erythrocytes and positive influence on immunocompetent organs. That is the reason of the treatment (especially joint diseases) with low–frequent laser rays (**Zubrod *et al.*, 2005; Scruton *et al.*, 2005**).

Table 3 Immune biological index in experimental animals

Indexes	Before treatment	After treatment
Experimental group 1		
T-lymphocytes (%)	47.00±1.45	57.00±0.79
B lymphocytes (%)	21.60±0.44	27.60±0.90
A immunoglobulins ($mg.ml^{-1}$)	1.40±0.05	2.60±0.07
M immunoglobulins ($mg.ml^{-1}$)	1.10±0.03	1.90±0.07
G immunoglobulins ($mg.ml^{-1}$)	12.60±0.24	18.10±0.58
Experimental group 2		
T-lymphocytes (%)	49.00±1.11	56.20±1.14
B lymphocytes (%)	20.00±0.79	27.00±0.79
A immunoglobulins ($mg.ml^{-1}$)	1.30±0.05	1.90 ±0.07
M immunoglobulins ($mg.ml^{-1}$)	1.00±0.03	1.40±0.05
G immunoglobulins ($mg.ml^{-1}$)	14.10±0.35	20.10±0.51
Experimental group 3		
T-lymphocytes (%)	51.00±1.00	52.20±0.74
B lymphocytes (%)	21.40±0.67	22.80±0.65
A immunoglobulins ($mg.ml^{-1}$)	1.20±0.03	
M immunoglobulins ($mg.ml^{-1}$)	13.70±0.60	13.80±0.45
G immunoglobulins ($mg.ml^{-1}$)	1.30±0.05	1.20±0.03

The joint diseases of animals have some regional peculiarities, connected with upkeep, feeding and exploitation. Thus, in most cases the reason of arthropathy of cattle and sheep is connected with application of cotton–plant fodders, containing poisonous polyphenol gossypol (**Ibragimov and Tovmasyan, 1973**; **Boymuradov *et al.*, 1986**; **Norboyev, 1990**; **Davlatov, 1993**). The beginning of the joint diseases depends on the peculiarities of their exploitation. If in most countries of the world horses are used in classic kind of sports, in our region they are widely used in national games: olmon poyga, kiz – kuv, kupkari and others, which are extremely popular in the Republics of Central Asia. These contests are held in all celebrities and cultural – mass measures (**Brubaker and Coss, 2015**).

It should be marked, that national games are held not always in specially equipped grounds. Very often they are held in the field conditions on the hard and stony ground. Besides these games consist of complex of complicated, hardly performed elements that is the reason why under such trauma – dangerous conditions the joint diseases of distal part of limbs of horses often appear (**Shakalov, 1952**; **Kuznetsov, 1980**; **Kalashnik and Peredara, 1988**; **Plakhotin *et al.*, 1991**).

The traumas are often complicated by affection of deep structures of the joint, sometimes with purulent inflammation, which leads to uncurable processes. In horses participating in sport and national games synovitis and periarticular fibrosis of joints of distal part of limbs are often diagnosed. One of unspecific influences on the organism is the application of the ray energy UVR, quantum, ultra–sound and especially low–frequent helium–neon laser rays (**Xu, 1985**; **Kana *et al.*, 1981**; **Mikaelyan, 1985**; **Izdepskiy and Rubenko 1987, 1989; Imanbayev and Nametov, 1992**;). At present auto-blood processed by laser for stimulation and treatment of the organism is injection by 3 methods – the external usage, internal application and extra carpal or parenteral injection. This method, besides stimulating influence has prolongated effect (**Xu, 1985**; **Skorina *et al.*, 1988**; **Izdepskiy and Rubenko 1989**) with data correlating to results found in this study. Injection of the auto-blood processed with laser was previously recommended (**Bessis and Ter-Pogossian, 1965**; **Rouns *et al.*, 1965**). Results are also well related to our data. It has been established that laser rays with the length of wave 632.8 nm have the most stimulating ability.

Results of this study in relation to used methods have scientific, practical and theoretical significance. Our clinical observations shown that 15% of animals taking part in classic and sport games and 22% of horses taking part in national sport games have limb diseases and especially of distal part. Besides, in the course of our researches there was ascertained, that the main reason of joint diseases are traumas, and that is why the chronic synovitis and periarticular fibrosis are more frequently developed. So our research was directed to the treatment of exact diseases. For the solution of this task for the first time we used methods of pathogenic therapy (pathogenic influence of laser rays) together with traditional methods, and also the specific preparation with mainly local influence. Clinical observations, morphological, biochemical, and also immunological indexes of blood we proved that the most effective method of treatment of joint diseases of distal part of limbs of horses with chronic synovitis and periarticular fibrosis is the application of parenteral injection of auto-blood, together with traditional methods and the application of "Hondralon" (2 ml intra articular) shortening the treatment to 5 days.

CONCLUSION

On the base of our experiments on elaboration the effective methods of treatment of aseptic forms of distal limb part inflammation in horses it can be concluded, that together with traditional method of treatment it is possible to use additionally effective pathogenic methods – to use auto-blood, processed with laser and also together with traditional method of treatment, auto-blood and administration of "Hondralon".

Together with clinical–morphological changes there were evident authentic changes of morphological, biochemical and immunological indexes, and also the pronounced immune stimulating influence of auto-blood, processed with laser and a positive influence of "Hondralon" on the physiological function of cartilage tissue was marked. On the base of conducted experiments there was ascertained that the most effective method of treatment of sport horses with the diagnosis of aseptic inflammation of distal limb part is the application of auto-blood processed with laser and "Hondralon" (2 ml intra jointly)

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CLONING AND EXPRESSION MOST EXPECTED ANTIGENIC FRAGMENT OF BETA-TOXIN GENE FROM *CLOSTRIDIUM PERFRINGENS* TYPE B

Maryam Gholami[1], *Mohsen Fathi Najafi*[*2], Mohammad Rabbani Khorasgani[3], *Behjat Majidi*[2]

Address(es):
[1]Department of Biotechnology, Faculty of advanced science and technologies, University of Isfahan, Hezarjarib St, 81746-73441, Isfahan, Iran. [2]Department of Veterinary Research and Biotechnology, Razi Vaccine and Serum Research Institute, Ahmadabad St, Mashhad, Iran. [3]Department of Biology, Faculty of Sciences, University of Isfahan, Hezarjarib St, 81746-73441, Isfahan, Iran.

*Corresponding author: m.najafi@mrazi.ac.ir

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ABSTRACT

Clostridium perfringens type B and C is an important pathogen and produces Beta-toxin which are responsible necrotic enteritis in humans or livestock. The death in individuals with this disease are over 50%. Vaccines against *C. perfringens* type B and C are currently manufactured using Beta-toxin produced by the virulent *C. perfringens* strain itself. To achieve the effective components for the creation of immunity at the first step used different primers in various location of Beta-toxin gene (cbp) by bioinformatics tools according to the secondary protein structure. After amplication of PCR products, one regions of Beta-toxin gene with high antigenicity was cloned into pTZ57RT and sub-cloned into the expression vector pET21a(+). The cloned vector was transformed into E. coli BL21 (DE3) and successfully expressed. Protein expression was confirmed by SDS-PAGE electrophoresis and western blotting. This recombinant peptide from most antigenic region of Beta-toxin gene can be suggested for antibody production and new peptide vaccine.

Keywords: *Clostridium perfringens*, Beta-toxin gene, Cloning

INTRODUCTION

Clostridia are ubiquitous and are commonly found in the environment, soil, decaying organic matter and a member of the gut flora in humans and animals **(Cato et al., 1986)**. *Clostridium perfringens* is an anaerobic, Gram-positive, rod-shaped, spore-forming bacterium that is one of most important pathogen of humans and livestock **(Miclard et al., 2009; Rood and Cole, 1991)**.
C. perfringens produces numerous toxin which are responsible for severve diseases inclusing intestinal or foodborne in human and animals. This micro-organism is classified into five toxintype (A, B, C, D and E) based on their ability to synthesize four major toxin, namely Alpha, Beta, Epsilon and Iota **(Petit et al., 1999)**. *C. perfringens* type B and C isolates, which produce Beta-toxin (BT) that causes necrotic enteritis in human and domestic animal **(Shatursky et al., 2000; Springer and Selbitz, 1999)**.
CBP is a lethal pathogenic factor of *C. perfringens* type B which aid in the lysis of HL-60 cells by forming cation-selective pores in the cell membrane **(Nagahama et al., 2003)**. This function is necessary for both necrotizing enteritis and lethal enterotoxemia caused by *C. perfringens* **(Nagahama et al., 2008; Sayeed et al., 2008)**. The gene for the BT has 1209 base pair with 336 amino acids in BT protein. The secreted toxin has similarities (based on 17% to 28% identity) to other toxins that are known to form pores in the plasma membranes of eukaryotic cells **(Hunter et al., 1993)**. Therefore, the production of toxins in heterologous expression systems is viable altenative.
The efficiency of vaccines based *C. perfringens* recombinant Beta-toxin has been reported. The importance of the Beta-toxin in human and animal diseases has been demonstrated by immunization studies with Beta toxoid. In one study α-β fusion gene from *C. perfringens* type C was cloned and expressed in E. Coli **(Bai et al., 2006)**. In this study the expressed α-β fusion protein can be used as the immunogens peptide for immunization. They constracted a recombinant epsilon-beta fusion protein for applying in vaccine production **(Langroudi et al., 2011)**. In another study, α/β2/β1 trivalent fusion-toxin (CPAB2B1) displayed increased immunogenicity relative to CPA and CPB2B1 alone. In other work, a vaccine based on Beta toxoid of *C. perfringens* type C produced and evaluated in E. Coli. The non-toxic recombinant Beta toxoid (rBT) was innocuous for mice and induced beta antitoxin in rabbits **(Milach et al., 2012).**
The aim of this work is production a recombinant fragment of Beta-Toxin (r-f-BT) from C. perfringenes type B in E. coli. Expected to use this recombinant protein for production of antibody against Beta-toxin of C. Perfringenes type B and futher applications.

MATERIAL AND METHODS

DNA extraction

Clostridium perfringens type B strain ATCC3626 prepared from Razi vaccine and serum research Institute. The cell was grown for 18 to 20 h at 37 °C in TGY (2% Trypticase, 2% glucose, 0.5% yeast extract). Genomic DNA was extracted by standard method with phenol/chloroform/isoamyl alcohol method **(Sambrook et al., 1989)**.

Primer designing

Since the aim of producing universal antibody for all antigenic components of Beta toxin *C. perfringens* therefore fragments of cpb were aligned and conserved gene sequences were selected. Multiple sequence alignments of 11 gene sequence of *C. perfringens* type B was performed using the CLUSTAL W2 program - (include: B-CPB240, B-CPB213, B-CPB228, B-CPB236, B-CPB220, B-CPB214, B-B, B-cpb, B-C-b, B-CWB-CN-228, B-CN301), we designed universal primers from conserved regions. Different primers were designed in various location of C.perfringens cpb gene (GenBank Accession No. X83275.1) according to the secondary structure of protein (Table 1). Secondary structure of beta protein (α-helix and β-sheet regions) was obtained based on the amino acid sequence of Beta toxin using PSIPRED Bioinformatics, then primers were designed according to different situations and out of range of the α-helix and β-sheet. Peptides outside the region of helix and loop regions are antigenic peptides. For confirmation of primer designing Immune Epitope Database (IEDB) was used.

Table 1 Six oligonucleotide primers for PCR-synthesizing cpb gene.

Primer name	Primer sequense	Sequence size	Position F-R
F1cbp	aaa gag caa tgt tca ttt aac tta aca	618 bp	1-618
R1cbp	tgt aga tga ttc agc ata ttc gct		
F2cbp	act aat tct act gca att aat ttt ccg	582 bp	399-981
R2cbp	gga ata gac ttg tcc tac cca gtt		
F3cbp	agc gaa tat gct gaa tca tct aca	474 bp	591-1065
R3cbp	aat agc tgt tac ttt gtg agt aag cca		

Polymerase chain reaction (PCR)

The PCR was carried out in a final volume of 50 µl containing 1 µg template DNA samples which were extracted from the bacterial strains. The target fragment was amplified using PCR Master Mix (Bioneer). A total of 35 cycles was performed under the following conditions: 94ºC for 5 min, and 1 cycles at 94ºC for 60 sec, 56ºC for 60 sec, 72ºC for 60 sec, then 1 cycles at 94ºC for 60 sec, 54ºC for 60 sec, 72ºC for 60 sec and then 35 cycles at 94ºC for 45 sec, 52ºC for 45 sec, 72ºC for 45 sec with a final extension at 72ºC for 10 min. PCR products were detected by 1.0% agarose gel electrophoresis and photographed.

"Cloning and expression r-f-BT protein"

The PCR product of Beta-toxin gene (cbp) with one region of cbp with high antigenicity (based on antigen prediction bioinformatics tools) was selected and extracted from the gel using the DNA recovery kit (bioneer). The extracted fragment was ligated into vector PTZ57RT (InsTAclone™ PCR Cloning Kit) according to manufacturers protocol. The recombinant plasmid was transformed into competent E. coli DH5α and selected on LB agar plates containing X-gal/IPTG and ampicillin. The white clones with positive plasmid were selected and controled by PCR using its specific primers and M13 primers. Plasmid digestion was performed by EcoR1 and Sal1 restriction endonuclease according to Fermentas protocol. After agarose electerophoresis, the EcoR1-cbp.f-Sal1 was purified and subcloned into pET21a(+) (Invitrogen) to generate the vector pET-21a-cbp.f. This transformant was picked and used to inoculate LB medium. The recombinant vector pET-21a-cbp.f was transformed into E. coli BL21(DE3) and selected by agar plate containing ampicillin and confirmed by restriction enzyme mapping. BL21 cells transformed with the plasmids described above were grown in LB medium with 100 µg/mL ampicillin at 37℃ to OD600 = 0.4~0.6. At this time, the expression of the protein was induced by adding 0.1 mM Isopropylthio-beta-galactoside (IPTG). The r-f-BT protein was purified (by instruction in www.thermo.com/pierce) and examined with SDS-PAGE and western blotting.

RESULTS

The aim of this project is to provide certain fragments of the Beta-toxin with high antigenicity and under epitope-focusing. First primers were designed based on the secondary structure with PSIPRED tool (Figure 1), then the results predicted antigenic regions of Beta-toxin by semi-empirical method showed that Beta-toxin has 9 antigenic regions which is shown in table 2. Thus region between 399−981 bp which has more than 5 antigenic regions of overlap with other fragments (Figure 2). Therefore this region was selected and tranformed.

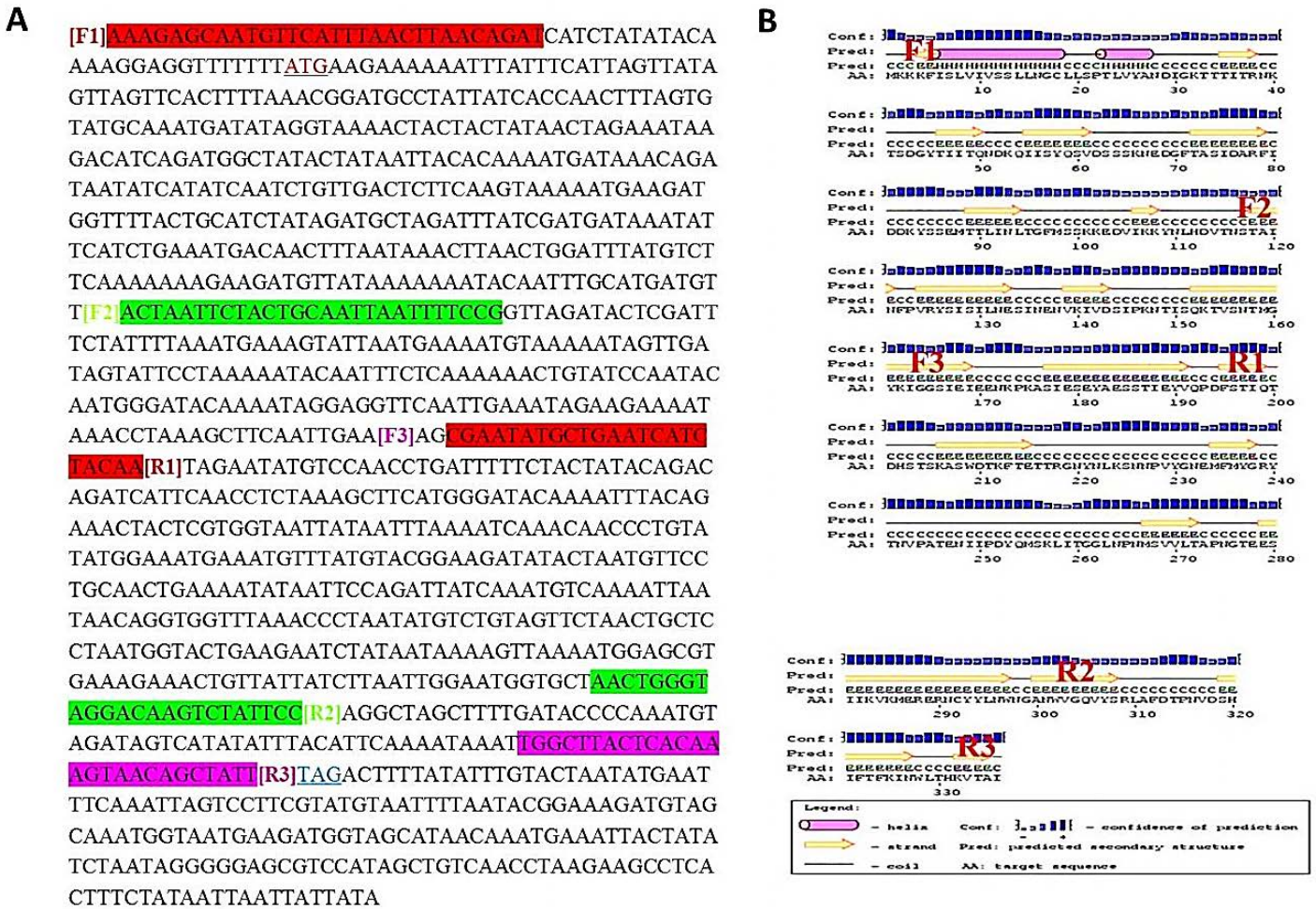

Figure 1 Bioinformatic tools for PCR Primer design **A.** Nucleotide sequence of Beta-toxin gene cpb from *C. perfringens* type B strain ATCC3626 and Suggested position for primers **B.** Primers was designed based on the secondary structure with PSIPRED tool.

Table 2 Predicted peptides of Beta-toxin by semi-empirical method

No.	Start Position	End Position	Peptide	Peptide Length
1	5	28	FISLVIVSSLLNGCLLSPTLVYA N	24
2	55	63	IISYQSVDS	9
3	103	114	EDVIKKYNLHDV	12
4	120	132	INFPVRYSISILN	13
5	138	145	NVKIVDSI	8
6	188	196	IEYVQPDFS	9
7	266	273	NMSVVLTA	8
8	303	314	VGQVYSRLAFDT	12
9	317	324	VDSHIFTF	8

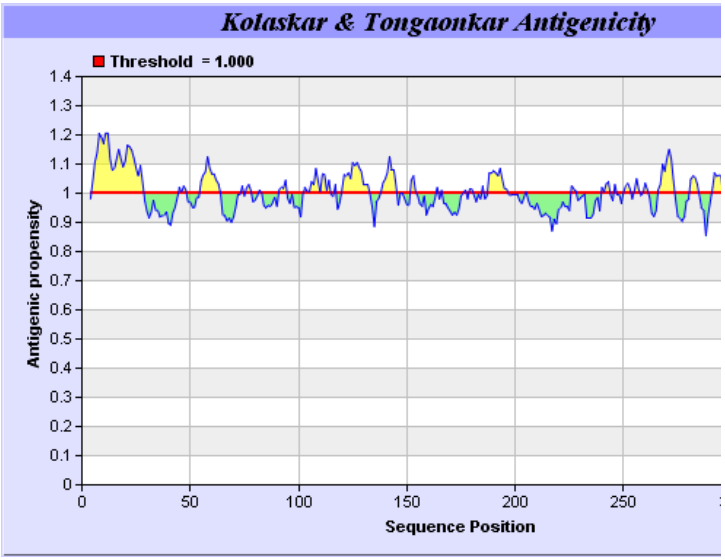

Figure 2 Semi-empirical method for prediction of antigenic regions of Beta-toxin *C. perfringens*.

"Gene cloning and expression of r-f-BT gene" in E. coli BL21 (DE3)

The r-f-BT gene from *C. perfringens* type B strain ATCC3626 was isolated from genomic DNA-extracted template by PCR amplification. The reaction yielded more products. Electrophoresis of PCR product confirmed the length of PCR fragment, which is shown in figure 3A. One of the fragments of Beta-toxin approximately 582 bp with high antigenicity was ligated to the cloning vector pTZ5R/T with T4 DNA ligase. The cloning vector containing the r-f-BT gene was introduced into competent E. coli DH5α cells by CaCl2 transformation. Transformed E. coli were grown at 37℃ in medium containing X-gal/IPTG and ampicillin. The positive plasmids were identified via sequential digestion with EcoR I and Sal I and r-f-BT protective antigen gene about 582 bp was obtained (Figure 3B).

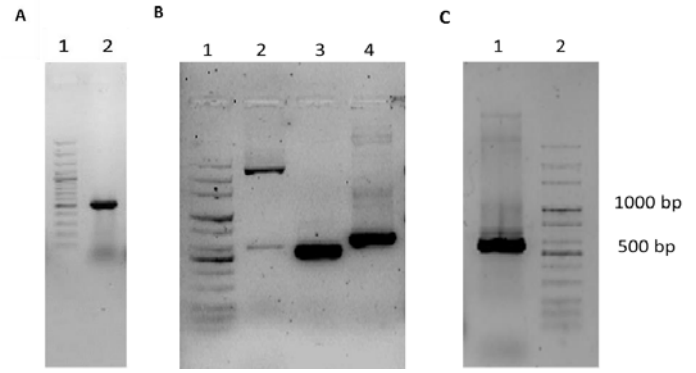

Figure 3 Agarose gel electrophoresis of r-f-β gene was amplified by PCR **A.** Lane 1: DNA molecular marker (GeneRuler 100 bp Plus DNA Ladder 100 to 3000 bp), Lane 2: 582bp. **In B.** Lane 1: DNA molecular marker. lane 2: pTZ57CPB/EcoR1 + Sal I. Lane 3, 4: Colony PCR results with Primer F2R2 (582bp) and M13 universal primers (741bp). **In C.** Cloned fragment into PET21 (a+) was confirmed by specific Primer F2R2.

The recombinant plasmid pET-21a-cbp.f was transformed into E. coli BL21(DE3) and the recombinant strain BL21(DE3) was obtained. Then SDS-PAGE and Brown band at the position of the reaction in western blotting confirmed the successful cloning and expression. The r-f-BT protein was produced in E. coli with an apparent molecular weight of 23 kD was observed (Figure 4).

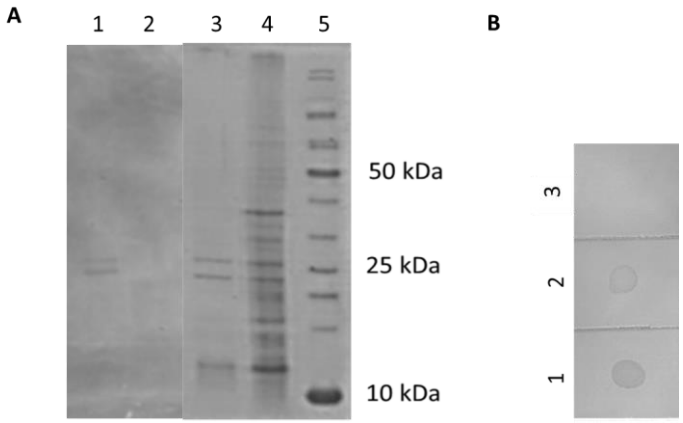

Figure 4 Characterization of r-f-BT producing in E. coli. **A.** Western blot and SDS-PAGE 12% analysis respectively, Lane 1: r-f-BT protein in reaction with native Beta-toxin antibodies. Lane 2: untransformed E. coli Bl21 (DE3) star extract (negative control). Lane 3: r-f-BT protein 23 kDa purified from E. coli inclusion bodies, Lane 4: Supernatant from soluble fraction of recombinant E. coli. Lane 5: Fermetas unstained protein marker. **B.** The results of dot blot analysis using native Beta-toxin antibodies. Lane 1: r-f-BT protein, Lane 2: BT *C. perfringens* (positive control), Lane 3: negative control.

DISCUSSION

Beta-toxin is one of the lethal toxins produced by *C. perfringens* type B and C strains **(Hunter et al., 1993)** Beta-toxin of *C. perfringens* (CBP) type B is caused the principal disease such as lamb dysentery in Great Britain and South Africa **(Niilo, 1980)**. Moreover, the toxoid vaccines majority of the commercial vaccines containing *C. perfringens* types B and C antigens, used in domesticated did not induce the minimum titers of b antitoxin, they are required to be tested for safety, residual toxicity and potency **(Milach et al., 2012;Titball, 2009)**.

Vaccine based on recombinant Beta toxoid (rBT) produced and evaluated in Escherichia coli, the non-toxic rBT was innocuous for mice and induced b antitoxin in rabbits. In other study the Beta-toxin gene fused to the glutathione-S-transferase (GST) was cloned and expressed in E. coli. The purified fusion protein is not toxic in mice and raised rabbit antisera against it specifically neutralises the toxic effect BT of *C. perfringens* type C culture filtrate in mice. Accordingly, the recombinant toxin proteins instead of their native toxins, are promising alternatives to the control of diseases caused by *Clostridium perfringens* **(Milach et al., 2012; Steinpórsdótti et al.,2006).** Other result suggested that recombinant toxoids are potential vaccine candidates against Clostridial toxins **(Zeng et al., 2011)**.

Due to the strong toxicity of Beta-toxin, we decided to evaluate a recombinant form of the toxin (rec-β) as a potential antigenic toxoid for production of a recombinant vaccine against C. perfringenes in future studies, after immunogenicity assay. As regards, the technology of recombinant protein antigens for immunization goes to identification main epitopes of protein antigens. Therefore antibody production is more successful with segments which contain epitope-focused antigens. The aim of this project is cloning a small fragment of the Beta-toxin with high antigenicity and epitope-focusing. First we designed primers according to the sequence and secondary structure of proteins that after protein structures are preserved**;** Beta-toxin has been shown more β-sheet than α–helix by secondary structure prediction. According to previous research, most pore-forming protein toxins have extensive β-sheet in their structure which can create suitable antigenic effect **(Parker and Feil, 2005)**. The variable regions were located in the external loop structures, while the predicted β-strands were formed by conserved sequences. The primers designing was done in external loop position. Epitope analysis plays an important role in the development of effective vaccine and diagnostic tools for different infection. In one study using different bioinformatics tools, one of the B cell epitopes of epsilon toxin comprising the region (Etx40-62) was identified. The rLTB.Etx40-62 fusion protein thus can be evaluated as a potential vaccine candidate against *C. perfringens* **(Kaushik et al., 2013)**.

In the present study we describes the successful isolation and cloning f-BT gene from strain of *C. perfringens*. We constructed a r-f-BT protein from *C. perfringens* type B. Ultimately western blot of r-f-BT protein showed that the antibodies specifically recognize antigen which it is attached. In summary, our findings demonstrate that r-f-BT of *C. perfringens* was capable of reacting with native Beta-toxin antibodies. The recombinant toxins with epitope-focused also be used to produce monoclonal antibody for immunoassay or possible therapy.

CONCLUSION

Herein, we reported that a r-f-BT of *C. perfringens* type B has been cloned and expressed in E. coli BL21, the achievement of this study was the production of r-f-BT with high antigenicity. These recombinant toxin (r-f-BT) proteins can replace the natural protein and can be used immunological detection of specific antibodies against the Beta toxin and vaccine research. These approaches were successful in maintaining the antigenicity of the epitope using bioinformatics tools, significantly minimize the time and efforts in generating recombinant protein with high antigenicity.

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