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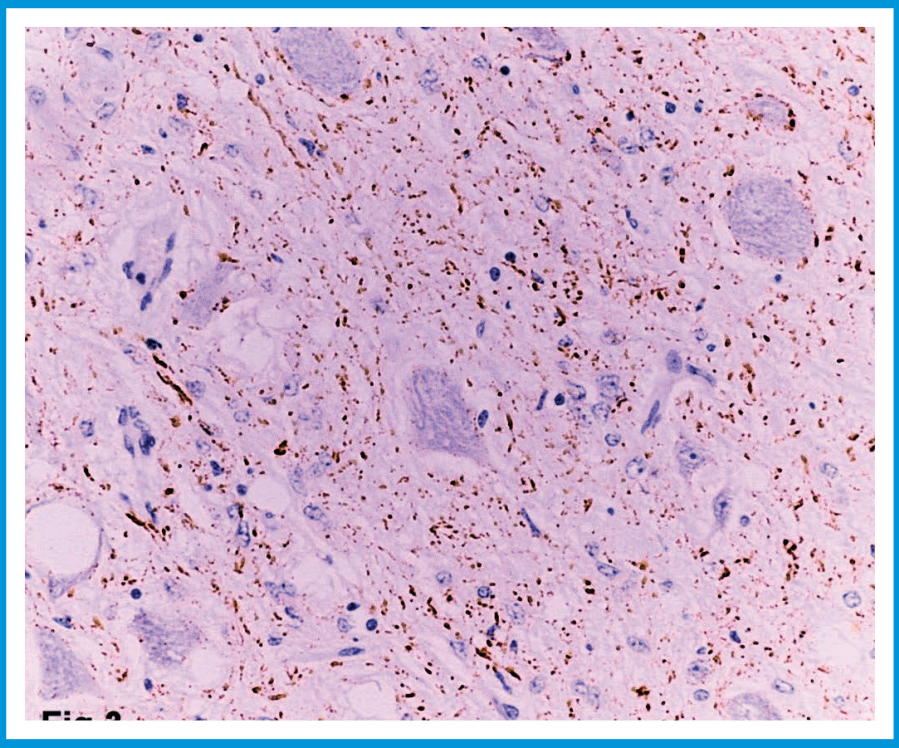

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Original Scientific Article

STUDY ON IMMUNE EFFICACY OF GENOMIC EXPRESSION LIBRARY VACCINES AGAINST AVIAN *PASTEURELLA MULTOCIDA*

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Summary: Random DNA fragments (500–3000 bp) were obtained from the genome of avian *Pasteurella multocida* CVCC474 using the restriction enzyme *Sau*3AI and cloned into the *Bam*HI site of the eukaryotic expression vector pcDNA3.1(+) to construct a genomic expression library. The library was subdivided into five clone pools and recombinant plasmids were extracted from each pool. Balb/c mice were allocated to groups 1–5 and vaccinated with these recombinant plasmids, whereas mice injected with pcDNA3.1(+) and PBS were used as negative controls. The immune responses were evaluated based on the serum antibody level, a lymphocyte proliferation assay, and the IFN - γ level. The protective efficacy after challenge with the virulent avian *Pasteurella multocida* strain CVCC474 was evaluated based on the relative protection rates. A significant increase in the serum antibody levels was observed in mice in group 1. The lymphocyte proliferation (SI value) and IFN - γ levels were also higher in mice immunized with the group 1 vaccine compared with those vaccinated with group 2–5 vaccines ($p < 0.05$) and control groups ($p < 0.01$). Furthermore, group 1 vaccine provided better protection than other vaccines after challenge with avian *Pasteurella multocida* strain CVCC474. The results indicated that construction of a genomic expression vaccine library is a promising approach for the prevention of avian pasteurellosis.

Key words: avian *Pasteurella multocida*; expression library; immunization; vaccine

Introduction

Avian *Pasteurella multocida* is the etiological agent of fowl cholera (avian pasteurellosis), which is a widely distributed disease that affects poultry, particularly chickens, turkeys, ducks, and geese, in many countries (1-3). The high morbidity and mortality associated with fowl cholera results in significant economic losses in the poultry industry (4). The main control measure used against this disease is drug treatment, particularly antibiotics such as streptomycin and olaquindox. However, the disease frequently recurs after drug withdrawal and the pathogen develops drug resistance after long-term medication, while it may also have significant toxic effects in avian species. The laying rate may be decreased significantly in layers while drug residues may persist in broilers. Therefore, it is necessary to develop novel vaccines for the control of fowl cholera.

Current vaccines for the prevention of fowl cholera include attenuated vaccines and inactivated vaccines. However, the immune efficacy with commercial live vaccines is not optimal. The attenuated vaccine has several shortcomings, such as a short period of immunity and poor protection. Residual virulence may lead

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to a reduction in the laying rate and there may be a disease outbreak if it is used incorrectly (5). The efficacy of the inactivated vaccine is not as high as the live vaccine, so the inactivated vaccine is used only rarely.

The lack of an effective vaccine that prevents this disease has stimulated the search for new candidate immunogens for novel vaccines. Expression library immunization (ELI) is an alternative approach with the potential to identify vaccine antigens (6-8). ELI was first reported by Barry (9). Piedrafita demonstrated that DNA vaccination with genomic libraries of *Mycoplasma pulmonis* could induce protection against challenge-exposure in parental strains of mice (10). Hernández showed that experimental animals immunized with a genomic expression library of *Mycobacterium tuberculosis* had a significant reduction in the number of viable bacteria in their lungs and less pulmonary tissue damage (11).

In this study, we constructed a genomic expression library of avian *Pasteurella multocida*. Recombinant plasmids were extracted and mice were vaccinated with these plasmids. The immune response and protective efficacy were tested. The aim of this study was to provide a foundation for the screening of antigens, and for further research and development into vaccines to combat avian *Pasteurella multocida* infection.

Materials and methods

Animals, bacterial strains, plasmids, and cells

Female Balb/c mice (6 weeks old) were purchased from the Experimental Animal Center in Henan Province. The avian *Pasteurella multocida* CVCC474 strain was purchased from the Chinese Institute of Veterinary Drug Control (IVDC). Competent cells (TG1) and the eukaryotic expression vector pcDNA3.1(+) were maintained in the laboratory at He Nan University of Science and Technology in China.

Construction of the genomic expression library

Genomic DNA was extracted from avian *Pasteurella multocida* CVCC474 strain as described previously (12) and digested with the restriction enzyme *Sau*3AI to produce median sizes of 500–3000 bp. These fragments were purified using a gel extraction kit. The eukaryotic expression vector pcDNA3.1(+) was digested with *Bam*HI and treated with alkaline phosphatase. It was ligated with the genomic DNA fragments and transformed into competent *E. coli* TG1 via electroporation (13). The *E. coli* cells were plated onto Luria-Bertani (LB) agar plates and incubated overnight at 37℃ to generate a genomic expression library containing about 10^6 clones.

Identification of the library and extraction of recombinant plasmids

Eight clones were selected randomly from the library and inoculated into 5 ml LB with ampicillin (50 μg/ml), before overnight culture at 37℃. The recombinant plasmids were extracted and identified using the restriction enzymes *Eco*RI and *Hind*III. The library was assigned randomly to five clone pools. The plasmids in each pool were prepared on a large scale using the alkaline lysis method and adjusted to 1 μg/μl using phosphate-buffered saline (PBS, 0.01 M, pH 7.2) for further experiments. These plasmids were referred to as DNA vaccines 1–5.

Vaccination protocol

Six-week-old female Balb/c mice (n = 168) were assigned randomly to seven groups, i.e., groups 1–5, the pcDNA3.1(+) group, and the PBS group. The mice in groups 1–5 and the pcDNA3.1(+) group were immunized with plasmids from clone pool groups 1–5 and the empty vector, pcDNA3.1(+), respectively. The vaccine dose was 100 μg/100 μl per injection for the plasmids from each clone pool and the pcDNA3.1(+). Mice in the PBS group were injected with 100 μl PBS (0.01 M, pH 7.2). The animals in each group were injected in both tibialis anterior muscles three times at 2 week intervals using 1 ml aseptic disposable syringes.

Detection of serum antibody levels

Blood samples measuring about 150 μl were drawn from mice by tail-cutting each week after immunization and the serum specimens were isolated. The serum antibody titers were detected

using an indirect enzyme-linked immunosorbent assay (ELISA). The ELISA was performed as follows. Flat-bottomed 96-well plates were coated with 100 µl ultrasound lysates (20 µg/ml) of avian *Pasteurella multocida* CVCC474. The plates were washed with 0.01 M PBS containing 0.05% tween-20 (PBS-T, pH 7.2) and blocked with 3% sodium caseinate for 2 h at 37 ℃. The plates were incubated for 1.5 h at 37 ℃ with 100 µl of the serum samples (diluted 1/100 with PBS-T). The plates were washed three times with PBST and goat anti-mouse IgG-horseradish peroxidase (HRP) was added and the plates were incubaed at 37 ℃ for 1.5 h. Then the plates were washed three times with PBST and 50 µl ortho-phenylene diamine (OPD) was added and the plates were incubated for a further 20 min. The enzyme activity was stopped by adding an equal volume of 2 M H_2SO_4 and the absorption was measured at 492 nm. The antibody titers were determined for up to 6 weeks before the challenge. All of the serum samples from each weekly collection were tested on the same ELISA plate, using two replicates.

Spleen lymphocyte proliferation assay of the immunized mice

Two weeks after each immunization, three mice were sacrificed from each group by cervical dislocation after administration of euthanizing drugs. The mice were soaked in 75% ethanol for 5 min. The spleen was removed via aseptic manipulation, before removing the fat and connective tissue, and washing with 1 ml Hanks' solution. The spleens were cut into 1–2-mm pieces and the tissues were blown fully with a pipette so that the cells were suspended in the Hanks' solution. The solution was then filtered through a three-tier gauze. Finally, the Hanks' solution containing the cells was collected and carefully layered onto the surface of lymphocyte separation medium. After centrifugation at 1000 x *g* for 20 min, the lymphocytes were collected and washed twice with RPMI-1640 without fetal bovine serum. The spleen lymphocyte suspension was resuspended and diluted to 1 x 10^7 cells/ml. An 50-µl aliquot of cell suspension was seeded into a 96-well plate (Greiner Bio-One, Longwood, Germany). Each experiment was repeated three times. Each well received 50 µl of 10 µg/ml ConA (experimental well) or 50 µl of RPMI 1640 medium (Gibco, Grand Island, USA) (negative control) and the plates were incubated at 37 ℃ in a 5% CO_2 environment. After 36 h, 10 µl of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich) was added to each culture well, followed by incubation for 3 h. After centrifugation for 10 min, the supernatant was discarded and 150 µl of dimethyl sulfoxide (DMSO) was added to the pellet, followed by incubation for 10 min until the crystals dissolved. The optical density (OD) of each well was measured using a microplate reader (Bio-Rad Laboratories, model 680) at a wavelength of 450 nm. The stimulation index (SI) was estimated using the following equation: SI = OD experimental well/OD negative control well.

IFN - γ assays

Two weeks after each immunization, the spleen lymphocytes activated by ConA were prepared as described above. The cells were incubated at 37 ℃ in 5% CO_2 for 60 h and the supernatants were harvested. The IFN-γ levels in the supernatants were detected using a commercial ELISA kit (Lianshuo, Shanghai, China), according to the manufacturer's instructions. The ELISA microtiter plate was coated with mouse IFN-γ monoclonal antibody which was combined with the IFN-γ in spleen lymphocyte supernatants. After the addition of anti-mouse IFN-γ antibody-horseradish peroxidase (HRP) and tetramet hylbenzidine (TMB), TMB became from colorless to blue under the catalysis of HRP and finally generated yellow, followed by the addition of H_2SO_4. The depth of color was directly proportional to the content of IFN-γ in samples. After the absorption was measured at 450 nm, the IFN-γ levels could be calculated according the standard curve. The measurement range of this mouse IFN-γ commercial ELISA kit was 0-1000pg/ml.

Challenge study

Fifteen days after the final vaccination, all of the mice were challenged subcutaneously with 3 x10^2 (5 LD_{50}) of virulent strain CVCC474. The mice were reared for 15 days and the survival number and relative protection rates were determined.

Statistical analysis

Data from all experiments were analyzed using the origin75 program. Analysis of variance was used to determine the significance of differences between the means in the experimental groups. Differences where $p<0.05$ were considered significant differences, while $p<0.01$ indicated highly significant differences.

Results

Identification of the library

Clones were randomly selected from the library and recombinant plasmids were extracted, before digesting with two restriction endonucleases, *Eco*RI and *Hind*III. The enzyme-digested products were analyzed by agarose gel electrophoresis. Figure 1 shows that DNA fragments between 500 and 3000 bp were obtained, indicating that the genomic expression library of avian *Pasteurella multocida* CVCC474 was constructed successfully.

Serum antibody levels

Conserved plates containing library clones were combined and assigned to five clone pools. The plasmids were extracted from each pool on a large scale and injected into mice. Serum specimens were isolated from vaccinated mice 1–6 weeks after the first immunization and assayed to determine the presence of specific antibodies using indirect ELISA. As shown in Figure 2, antibody levels of serum from all the experimental groups (groups 1–5) exhibited an increasing trend after immunization. The serum antibody levels in group 1 were significantly higher than those in groups 2–5 ($p < 0.05$) and the two negative control groups, i.e., the pcDNA3.1(+) group and the PBS group ($p < 0.01$). There were no differences in the antibody levels among the other experimental groups ($p > 0.05$), although group 2 was slightly higher than the others. In addition, the serum antibody levels in groups 1–5 were significantly higher than those in the pcDNA3.1(+) group ($p < 0.01$).

Spleen lymphocyte proliferation assay

To investigate the cellular immune response induced by the genomic expression library, we assessed the proliferation of spleen lymphocytes at three different time points after each vaccination experiment and the results are shown in Figure 3. In each experiment, the SI values of groups 1 and 4 were higher than that of other groups. After the first immunization, the SI values of groups 2–5 were similar to that of group 1 ($p > 0.05$). After the second and the third immunizations, however, the SI value of group 1 was significantly higher than that of groups 2–5 ($p < 0.05$) and the negative control groups ($p < 0.01$). There were also no differences among groups 2–5 ($p > 0.05$), although group 4 was slightly higher than the others.

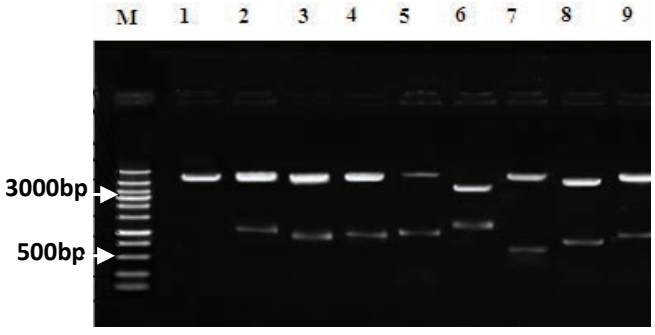

Figure 1: Electrophoresis of recombinant plasmids digested with *Eco*RI and *Hind*III. M: wide range DNA marker (100–6000); 1: pcDNA3.1(+) empty plasmid; 2–9: recombinant plasmids digested using *Eco*RI and *Hind*III

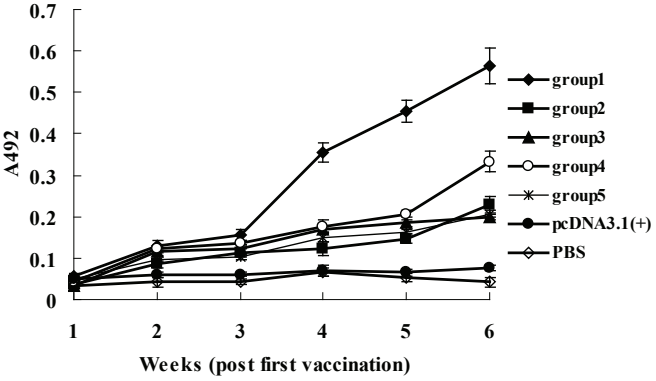

Figure 2: Dynamic changes in the serum antibody levels of immunized mice. The serum antibody levels were measured by indirect ELISA weekly, until 6 weeks. Mice were immunized with group 1 vaccine (■); group 2 vaccine (●); group 3 vaccine (▲); group 4 vaccine (▼); group 5 vaccine (◆); pcDNA3.1(+) vector (○); and PBS (△)

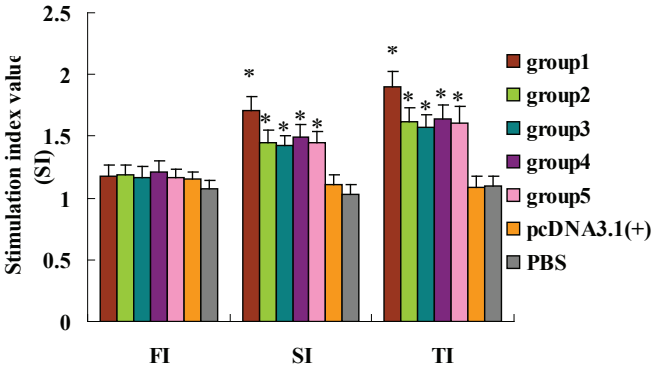

Figure 3: MTT detection of splenic lymphocyte proliferation in immunized mice. ConA was administered to stimulate mouse splenic lymphocytes 2 weeks after each immunization. FI: 1 week after the first immunization; SI: 1 week after the second immunization; TI: 1 week after the third immunization. *$p < 0.05$, **$p < 0.01$

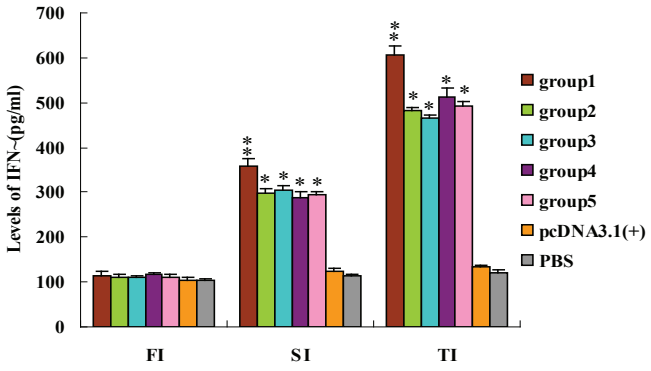

Figure 4: Analysis of mouse splenic lymphocyte-secreted IFN~γ levels. The results are expressed as the mean ± S.D. FI: first immunization; SI: second immunization; TI: third immunization. *$p < 0.05$, **$p < 0.01$

IFN~γ secretion

After ConA stimulation, the IFN~γ levels secreted by mouse splenic lymphocytes were higher in mice vaccinated with all recombinant plasmids (groups 1–5). After the first immunization, there were no significant differences in the IFN~γ levels ($p > 0.05$). After the second and third immunization, the IFN~γ level in group 1 was significantly higher than that in other groups ($p < 0.05$). The levels were similar among groups 2–5, but higher than in the two control groups ($p < 0.05$) (Figure 4).

Protection against challenge with avian Pasteurella multocida CVCC474

Groups of mice were challenged with the live virulent avian *Pasteurella multocida* CVCC474 strain 15 days after the last immunization. The survival number and relative protection rates were calculated up to 15 days (Table 1). The mortality of mice injected with PBS was 93.33% after challenge whereas that of the pcDNA3.1(+) group was 86.67%. The relative protection rates in groups 1 and 4 were higher than in the other groups ($p < 0.05$). The protective efficiency in group 1 was the highest (64.29%).

Table 1: Protective efficacy against lethal challenge with *avian Pasteurella multocida*

Groups	Survival number/total	Mortality(%)	Relative protection rate (%)
group 1	10/15	33.33	64.29**
group 2	5/15	66.67*	28.57*
group 3	3/15	80.00**	14.29
group 4	7/15	46.67*	50.00*
group 5	5/15	66.67*	28.57*
pcDNA3.1(+)	2/15	86.67**	0
PBS	1/15	93.33**	0

Relative protection rate (%) = (1 - mortality of experimental groups/mortality of control group) x 100%. *$p < 0.05$, **$p < 0.01$.

Discussion

Current candidate antigens for avian *Pasteurella multocida* DNA vaccines include outer membrane proteins (Omps), capsules, and Type 4 fimbriae (14-16). However, there are large differences in the immune efficacy of DNA vaccines constructed using these antigen genes. In general, the protective efficacy of these DNA vaccines rarely exceeds that of attenuated live vaccines (17). Thus, the screening of novel protective antigen genes is necessary to develop effective vaccines for controlling avian pasteurellosis. Immunization with genomic expression libraries has emerged as a novel technology (18, 19) for identifying candidate vaccine genes that provide protection against pathogens. Some studies have identified individual protective genes via the sequential fractionation of cDNA or genomic expression libraries (20-23). The identification of vaccine candidates to combat avian *Pasteurella multocida* using this technology is promising because another study demonstrated that fusion and combined DNA vaccines against avian pasteurellosis could induce a higher immune response than monovalent DNA vaccines (24).

In the present study, we constructed a genomic expression library of avian *Pasteurella multocida* and evaluated its immune efficacy. The humoral immune response is an important factor in the resistance to avian *Pasteurella multocida* infections. In this study, we detected the antibody levels induced by DNA vaccines extracted from a library of clone pools in mice (group 1–5) and we found that the antibody response induced by DNA vaccines extracted from clone pool group 1 was higher compared to other vaccines.

In addition to the antibody response, the cellular immunological response also has an influential role in the anti-infection process (25, 26). It is well-known that DNA vaccines can induce effective immune responses (27, 28). Lymphocyte proliferation assays and the detection of cytokine secretions are commonly used methods for evaluating the cellular immune function. The lymphocyte transformation rate after stimulation by ConA is frequently used to evaluate the T-cell immune response (29). In this study, the DNA vaccine prepared from clone pool group 1 induced higher lymphocyte proliferation levels and higher IFN~γ secretion from immunized mice compared with the other groups.

Challenge experiments are one of the important indices used to evaluate the protective efficacy of vaccines. In the present study, mice immunized with various recombinant plasmids were challenged with virulent avian *Pasteurella multocida*. Of these DNA vaccines, the plasmids from clone pool group 1 showed the most promise.

Future directions for our studies could include investigating Th2 cytokines, such as IL-4 and IL-10, which have been shown to modulate the immune responses to DNA vaccines (30, 31). The next task is to identify specific antigens in the library, which can be prepared as subunit and DNA vaccines to induce a protective immune response against fowl cholera. In conclusion, we demonstrated that immunization with an expression library enabled the screening of the avian *Pasteurella multocida* genome for potential vaccine candidates, which provided a valuable reference for the design of future DNA vaccines against fowl cholera.

Acknowledgements

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References

1. Davies RL, MacCorquodale R, Caffrey B. Diversity of *avian Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. Vet Microbiol 2003; 91: 169–82.

2. Hussam AA, Takuo S, Hitoshi H, Yoshiya K, Noriyuki O, Osamu I. Invasion of chicken embryo fibroblast cells by *avian Pasteurella multocida*. Vet Microbiol 2004; 104: 55–62.

3. Shivachandra SB, Kumar AA, Gautam R, Singh VP, Saxena MK, Srivastava SK. Identification of avian strains of *Pasteurella multocida* in India by conventional and PCR assays. Vet J 2006; 172: 561–4.

4. Subaaharan S, Blackall LL, Blackall PJ. Development of a mμlti-locus sequence typing scheme for avian isolates of *Pasteurella multocida*. Vet Microbiol 2010; 141: 354–61.

5. Mariana S, Hirst R. The immunogenicity and pathogenicity of *Pasteurella multocida* isolated from poultry in Indonesia. Vet Microbiol 2000; 72: 27–36.

6. Kamath AT, Feng CG, Macdonald M, Briscoe H, Britton WJ. Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. Infect Immun 1999; 67:1702–7.

7. Liu SG, Gong Q, Wang C, et al. A novel DNA vaccine for protective immunity against virulent *Mycobacterium bovis* in mice. Immunol Lett 2008; 117: 136–45.

8. Geza E, Sameera S, Patricia A, et al. Development and characterization of a pooled *haemophilus influnzae* genomic library for the evaluation of gene expression changes associated with mucosal biofilm formation in otitis media. Int J Pedia Otorhinolar 2003; 67: 749–55.

9. Barry MA, Laiand WC, Johnston S. Protection against *mycoplasma* infection using expression library immunization. Nature 2002; 377: 632–35.

10. Piedrafita D, Xu D, Hunter D, Harrison RA, Liew FY. Protective immune responses Induced by vaccination with an expression genomic library of *Leishmania major*. J Immunol 1999; 163: 1467–72.

11. Hernández YL, Corona DY, Rodríguez SS, et al. Immunization of mice with a *Mycobacterium tuberculosis* genomic expression library results in lower bacterial load in lungs after challenge with BCG. Tuberculosis 2006; 86: 247–54.

12. Moore RJ, Lenghaus C, Sheedy SA, Doran TJ. Improved vectors for expression library immunization-application to *Mycoplasma hyopneumoniae* infection in pigs. Vaccine 2001; 20: 115–20.

13. Tollefsen S, Vordermeier M, Olsen I, Storset AK, Reitan LJ, Clifford D. DNA injection in combination with electroporation: a novel method for vaccination of farmed ruminants. Scand J Immunol 2003; 57: 229–38.

14. Entomack B, Sawada T, Kataoka Y, Ohtsu N, Akagi M, Nakamura SA. 39KDa protein mediates adhesion of avian *Pasteurella multocida* to chicken embryo fibroblast cells. Vet Microbiol 2003; 97: 229–43.

15. Prado ME, Dabo SM, Confer AW. Immunogenicity of iron-regulated outer membrane proteins of *Pasteurella multocida* A:3 in cattle: molecular characterization of the immunodominant heme acquisition system receptor (HasR) protein. Vet Microbiol 2005; 105: 269–80.

16. Hatfaludi T, Al-Hasani K, Boyce JD. Outer membrane proteins of *Pasteurella multocida*. Vet Microbiol 2010; 144: 1–17.

17. Yamile LH, Daniel YC, Sergio SR, et al. Immunization of mice with a *Mycobacterium tuberculosis* genomic expression library results in lower bacterial load in lungs after challenge with BCG. Tuberculosis 2006; 86: 247–54.

18. Alberti E, Acosta A, Sarmiento ME, et al. Specific cellular and humoral immune response in Balb/c mice immunised with an expression genomic library of *Trypanosoma cruzi*. Vaccine 1998; 16: 608–12.

19. Yero CD, Pajón FR, Caballero ME, et al. Immunization of mice with *Neisseria meningitidis* serogroup B genomic expression libraries elicits functional antibodies and reduces the level of bacteremia in an infant rat infection model. Vaccine 2005; 23: 932–9.

20. Melby P, Ogden G, Flores H, et al. Identification of vaccine candidates for experimental visceral leishmaniasis by immunization with sequential fractions of a cDNA expression library. Infect Immun 2000; 68: 5595–602.

21. Lvey FD, Magge DM, Woitaske MD, Johnston SA, Cox RA. Identification of a protective antigen of *coccidioides immitis* by expression library immunization. Vaccine 2003; 21: 4359–67.

22. Halea S, Kaltenboeckb B, DeGravesb FJ, et al. Screening the whole genome of a pathogen in vivo for individual protective antigens. Vaccine 2005; 23: 3016–25.

23. Beltrán PK, Gutiérrez OA, Puebla PA, et al. Identification of immunodominant antigens of *Mycobacterium bovis* by expression library immunization. Vet J 2010; 1: 181–3.

24. Gong Q, Cheng M, Niu MF. Out membrane protein DNA vaccines for protective immunity against virulent avian *Pasteurella multocida* in chickens. Procedia Environ Sci 2011; 8: 723–9.

25. Liao CM, Huang CJ, Hsuan SL, et al. Immunogenicity and efficacy of three recombinant subunit *Pasteurella multocida* toxin vaccines against progressive atrophic rhinitis in pigs. Vaccine 2006; 24: 27–35.

26. Register KB, Sacco RE, Brockmeier SL. Immune response in mice and swine to DNA vaccines derived from the *Pasteurella multocida* toxin gene. Vaccine 2007; 25: 6118-6128.

27. Lee CG, Choi SY, Park SH, Park KS, Ryu SH, Sung YC. The synthetic peptide Trp-Lys-Tyr-Met-Val-d-Met as a novel adjuvant for DNA vaccine. Vaccine 2005; 23: 4703–10.

28. Herrmann JE. DNA vaccines against enteric infections. Vaccine 2006; 24: 3705–8.

29. Stone KD, Feldman HA, Huisman C,

Howlett C, Jabara HH, Bonilla FA. Analysis of in *vitro* lymphocyte proliferation as a screening tool for cellular immunodeficiency. Clin Immunol 2009; 131: 41–9.

30. Huang Y, Babiuk LA, Hurk SD. The cell-mediated immune response induced by plasmid encoding *bovine herpesvirus* 1 glycoprotein B is enhanced by plasmid encoding IL-12 when delivered intramuscularly or by gene gun, but not after intradermal injection. Vaccine 2006; 24: 5349–59.

31. Williman J, Lockhart E, Slobbe L, Buchan G, Baird. The use of Th1 cytokines, IL-12 and IL-23, to modulate the immune response raised to a DNA vaccine delivered by gene gun. Vaccine 2006; 24: 4471–4.

ŠTUDIJA IMUNSKE UČINKOVITOSTI CEPIV PROTI AVIARNI *PASTEURELLA MULTOCIDA* NA OSNOVI GENOMSKE KNJIŽNICE

Q. Gong, M. Cheng, M. Niu, C. Qin

Povzetek: Genomsko ekspresijsko knjižnico smo naredili z naključnimi odseki DNK (500-3000 bp), ki so bili pridobljeni iz genomske DNK ptičje bakterije *Pasteurella multocida* CVCC474 z restrikcijsko razgradnjo z encimom Sau3AI. Odseki so bili klonirani v mesta BamHI vektorja za izražanje v evkariontskih celicah pcDNA3.1(+). Knjižnica je bila razdeljena na pet delov in iz vsakega smo izolirali rekombinantne plazmide. Miši seva Balb/c smo razdelili v pet skupin in jih cepili s 5 seti rekombinantnih plazmidov, kontrolni skupini pa smo vbrizgali prazen vektor pcDNA3.1 (+) v slanem fosfatnem pufru. Imunski odgovor smo ocenili na osnovi serumske ravni protiteles, s testom proliferacije limfocitov in ravni IFN-γ.

Učinkovitost zaščite po okužbi z virulentnim sevom CVCC474 ptičje bakterije *Pasteurella multocida* smo ocenili na osnovi relativnih zaščitnih meril. Pri miših v skupini 1 smo opazili značilen dvig serumske ravni protiteles. Prav tako smo pri skupini 1 opazili statistično značilno povečanje proliferacije limfocitov in IFN-, tako v primerjavi s kontrolno skupino ($p < 0,01$) kot s testnimi skupinami 2 do 5 ($p < 0,05$). Rezultati so pokazali, da je cepivo v obliki genomske ekspresijske knjižnice obetaven pristop za preprečevanje ptičje pastereloze.

Ključne besede: ptičja *Pasteurella multocida*; ekspresijska knjižnica; cepljenje; cepivo

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Original Scientific Article

CLONING OF BASIC FIBROBLAST GROWTH FACTOR FROM CHINESE SMALL TAIL HAN SHEEP AND ITS EFFECTS ON PROLIFERATION OF MURINE C2C12 MYOBLASTS

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Summary: To further understand characteristics and functions of basic fibroblast growth factor (bFGF, FGF-2) as well as its effects on skeletal muscle, a coding sequence of 18kDa-bFGF gene was cloned from Chinese Small Tail Han sheep, an excellent indigenous animal breed in China. The DNA sequence and deduced protein sequence were analyzed and aligned with the counterparts of other published animals. The results indicated that most hallmarks of the newly cloned bFGF gene sequence were found to be similar to those of published species, which implied further that bFGF genes have been conservative in evolution. The complete coding sequence for the gene was inserted into pET-28a plasmid and expressed predicted fusion protein in E. coli. The product was soluble and possessed of biological activity, it is sure to be a good source for further investigation. In addition, effects of the products on proliferation of murine C2C12 myoblasts were investigated. The findings demonstrated that the ovine recombinant bFGF accelerated in vitro the proliferation of murine C2C12 myoblasts, and MEK/ERK-MAPK signaling pathway participated in the regulative mechanism.

Key words: bFGF; cloning; C2C12; MEK/ERK; Small Tail Han sheep

Introduction

Basic fibroblast growth factor(bFGF, FGF-2) is a member of the FGF family, which comprises twenty-two members that are structurally related polypeptide growth factors found in organisms ranging from nematodes to humans and encoded by twenty-two distinct genes in mouse(1). The protein is a single-chain polypeptide composed of 146 amino acids firstly purified from bovine pituitary. Five forms of the protein, including 18,22,22.5,24 and 34kDa, possessing of identical C-terminal sequences, result from different initiation codon of the same transcript. The 18kDa form is produced by initiation of translation at AUG codon and all the other four forms come from initiation of translation at CUG codons located 5' upstream from the AUG codon. Generally, 22,22.5,24,34 kDa-bFGF are designated as high molecular weight (HMW) forms bFGF (2,3). The 18kDa-bFGF is localized primarily in the cytoplasm whereas the HMW-bFGFs are predominantly localized in the nucleus (4), which imply that they may perform different biology roles through respective signal pathways.

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Basic FGF was initially identified as a mitogen with prominent angiogenic properties, but now was recognized as a pleiotropic effector in different cells and organ systems (5), a key function is the ability to stimulate proliferation of cells derived from mesodermal and neuroectodermal tissues (6), and found in all organs, solid tissues, tumors and cultured cells including muscle cells (7-12). Basic FGF is regarded as a potent stimulator of the proliferation and fusion of myoblasts in vitro and a factor to enhance muscle regeneration in vivo (13,14). Impaired expression of endogenous bFGF gene in rat skeletal muscle caused by ischemia and reperfusion may be a reason in delayed wound healing (15,16). Furthermore, it has been reported that there is a positive relationship between bFGF and the speed and success of muscle regeneration (17). During the skeletal muscle course of development and regeneration, many growth factors exert their biological roles, for example, bFGF and myostatin, the former improves proliferation of C2C12 myoblasts and the latter plays an opposite role. Our previous reports revealed the suppression of bFGF on endogenous expression of myostatin gene in C2C12 myoblasts and the participation of ERK/MEK-MAPK signaling pathway in the regulatory process(18,19).

It has been proved that bFGF is highly conservation among all published species and performs biological roles on development, wound repair and regeneration of skeletal muscle. To understand the evolution of bFGF on a deeper time scale and effects on skeletal muscle, in this paper we report the molecular cloning and characterics of 18kDa-bFGF orthologous genes expressed in Chinese Small Tail Han sheep (CSTHS), a famous indigenous Chinese sheep breed, and expression and purification of the protein overproduced in *E.coli* (BL21). In addition, effects and regulative mechanism of bFGF on proliferation C2C12 myoblasts are also discussed.

Materials and methods

PCR amplifys complete 18kDa-bFGF gene of Small Tail Han sheep

Total RNA of CSTHS was isolated from spleen preserved in author's laboratory with Trizol reagent according to the manufacturer's protocol. For the amplification of 18kDa-bFGF gene, a set of primers was synthesized based on our published CSTHS 18kDa-bFGF cDNA sequence (GenBank accession numbers: DQ091182). 5'-GATATCATGGCCGCCGGGAGCATCA-3' and 5'-GTCGACTCAGCTCTTAGCAGACATTGG-3' were chosen as the sense primer and antisense primer. The PCR products were ligated into pMD18-T vector and the ligated products were used for transforming *E.coli* DH5α competent cells. The recombinant plasmid DNA, pMD18-T/obFGF, was isolated and sequenced, and protein putative structure of bFGF was deduced according to DNA sequences. DNA and protein sequences were compared with the counterparts of other published species. Database searches and sequence alignment were performed at the National Center for Biotechnology Information and with DNAMAN Software.

Construction of expression plasmid

To construct an expression vector, a set of primers 5'-ATGAATTCATGGCCGCCGGGAGCATCA-3' carrying an *EcoRI* site and 5'-GCACTCGAGAGCTCTTAGCAGACAT-3' containing an *XhoI* site were employed to amplify bFGF complete coding seuqence from plasmid pMD18-T/obFGF. The amplification products were digested with *EcoRI* and *XhoI* endodeoxyribonuclease, and then ligated into vector pET-28a that had been previously cut with the same enzymes, the final vector pET-28a/obFGF was resulted. DNA of pET-28a/obFGF was produced by transforming into *E.coli* DH5α and transformed into *E.coli* BL21. The sequence of the insert was confirmed using automated DNA sequencing.

Expression and purification of ovine recombinant bFGF(or-bFGF)

E.coli cells harboring pET-28a/obFGF were grown in 100 ml of LB medium containing kanamycin at 37℃. When the A600 reached 0.6-0.8, the culture was added to 1 L of LB medium containing kanamycin and incubated at 37℃. When the A600 reached 0.6-0.8 again, isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5mM and the culture was continued at 25℃ for 5 h. Cells were harvested by centrifugation at 5000g for 15min at 4℃ and the cell pellet was washed with ice-cold binding buffer

containing 20mM sodium phosphate and 500mM sodium chloride adjusted to pH7.8 with H_3PO_4. Cells were resuspended in 20ml of binding buffer containing 1mM PMSF and 1mg/ml lysozyme and incubated at 4℃ for 30min. The suspension was sonicated and then centrifuged at 12000g for 20min at 4℃. The supernatant was harvested and applied to a Ni-NTA column equilibrated with binding buffer. The column was washed with washing buffer(20mM sodium phosphate, 500mM sodium chloride) adjusted to pH6.0 with H_3PO_4 and supplemented with 20mM imidazole, until the A280 was less than 0.01. Proteins were eluted with elution buffer containing 20mM sodium phosphate, 500mM sodium chloride and 250mM imidazole. Fractions containing most of the A280 material were pooled. The purity of or-bFGF in column fractions was assessed using SDS-PAGE. The purified protein was dialysed in dialysis buffer (5mM Tris-HCl, pH7.6). Protein concentration was determined by bicinchoninic acid assay. The protein was freeze-dried and stored at -80℃.

Immunoblotting analysis

Protein samples were denatured in sample buffer for 5 min in boiling water and loaded onto SDS-PAGE. Electrophoresis was carried out before the protein was transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween-20. Then, the membranes were incubated with polyclonal antibody against human bFGF for 1hr. Secondary antibody conjugated with horseradish peroxidase was incubated after washing with TBS containing 0.05% Tween-20 for 1 h. The blot was washed four times and colored using DAB kit according to the manufacturer's protocol.

Activity assay of fusion protein

The biological activity of or-bFGF protein was detected by the induction of proliferation of 3T3 fibroblasts. NIH-3T3 fibroblasts diluted to 1×10^5 / ml were grown in DMEM supplemented with 10% fetal bovine serum(FBS). When the cells was about 80% confluent, cells were transferred into a 96-well plate and incubated in DMEM supplemented with 10% FBS for 8 h, with five replicates of each well, then the medium was changed to DMEM supplemented with 0.5% FBS and incubated for 48 h. Cells were incubated in medium (containing different concentrations of either human recombinant bFGF(hr-bFGF, Sigma) or or-bFGF, 10ng/ml heparin, and 0.5%FBS) for 48 h, methylthiazoletetrazolium solution in concentration of 5mg per ml was added into wells. After 6hr, each well was added with 100µl DMSO. The plate was vibrated for 20min on a vibrator. Optical density values were read in enzyme-linked immunosorbent assay instrument, at wavelength 570nm.

Murine C2C12 myoblasts proliferation assay

Murine C2C12 myoblasts were grown to 80% confluence in DMEM with 10% FBS. Cells were harvested, diluted to 1×10^5 /ml, added in quintuplicate to each well of 96-well tissue culture plate and incubated for 24 h. Medium was replaced with DMEM containing 0.5% FBS and incubated for 48 h. The medium was replaced with DMEM containing 0.5% FBS, different concentrations of or-bFGF and 10ng/ml heparin. The following incubation was performed for 48 h and MTT was added into wells. Followed incubation for 6 h, wells were added with 100µl of DMSO and sufficiently vibrated for 20min. Optical density values at wavelength 570nm were measured.

Effects of inhibitors of MAP kinase signaling pathways on proliferation of C2C12 myoblasts induced by bFGF

C2C12, grown to 80% confluence in DMEM with 10% FBS, were plated in quintuplicate into each well of 96-well tissue culture plate at a density of 1×10^5 /ml and incubated for 24 h. Myoblasts were transferred to DMEM with 0.5% FBS and incubated for 48 h. Cells were pretreated for 1h with medium containing 0.5% FBS and inhibitor (10µM SB203580, 50µM PD98059 or 10µM SP600125). Cells were transferred into medium containing 0.5% FBS, 20ng/ml or-bFGF and 10ng/ml heparin. The following incubation was performed for 48 h. MTT assay was employed to detect cell proliferative activity.

Data and statistical analysis

Experimental data were analyzed by ANOVA using the SPSS statistical software. Results were expressed as the means and standard errors. Significance was considered at $p<0.05$ or $p<0.01$.

Results

Molecular characteristics

Nucleotide sequence for the coding region of bFGF cDNA from Small Tail Han sheep is different to the other published sequences, but all known 18kDa-bFGF orthologous gene sequences remain highly conservation during evolution. The similarities of coding nucleotide sequences between Small Tail Han sheep and other published terrestrial animals were found to be 99.8% (cattle, NM174056), 98.9% (sheep, NM001009769), 95.3% (chimpanzee, AY665259), 95.1% (human, NM002006; monkey, XM001099284), 94.2% (dog, XM533298), 90.8% (house mouse, NM008006), 89.5% (Norway rat, NM019305), 87.4% (gray short-tailed opossum, NM001033976), and 84.8% (non-mammalian chicken, NM205433) respectively. The similarities between the sheep and other published amphibian were 78.0% (Japanese firebelly newt, AB064664) and 75.9% (silurana tropica, NM001017333), and the similarities between the sheep and other published aquatic animals were 69.7% (zebrafish, NM212823) and 67.9% (rainbow trout, AY878375).

Deduced amino acid sequence of 18kDa-bFGF from Small Tail Han sheep was aligned with NCBI and DNAMAN software. The results showed that polypeptide sequences among all species were conservative and the similarities of Small Tail Han sheep protein primary structure to that of other published species were 99.4% (cattle), 98.7% (dog, sheep), 98.1% (human, chimpanzee and monkey), 97.4% (Norway rat), 95.5% (house mouse), 94.2%(gray short-tailed opossum), 93.5% (chicken), 86.5% (Japanese firebelly newt), 82.5% (silurana tropica), 74.7% (zebrafish) and 74.2% (rainbow trout), respectively. Nucleotide and amino acid sequences were given in Figure 1.

```
1     ATG GCC GCC GGG AGC ATC ACC ACG CTG CCA GCC CTG CCG GAG GAC GGC GGC GGC GGC GCT
1      M   A   A   G   S   I   T   T   L   P   A   L   P   E   D   G   G   G   G   A
61    TTC CCG CCG GGC CAC TTC AAG GAC CCC AAG CGG CTG TAC TGC AAG AAC GGG GGC TTC TTC
21     F   P   P   G   H   F   K   D   P   K   R   L   Y   C   K   N   G   G   F   F
121   CTG CGC ATC CAC CCC GAC GGC CGA GTG GAC GGG GTC CGC GAG AAG AGC GAC CCA CAC ATC
41     L   R   I   H   P   D   G   R   V   D   G   V   R   E   K   S   D   P   H   I
181   AAA CTA CAA CTT CAA GCA GAA GAG AGA GGG GTT GTG TCT ATC AAA GGA GTG TGT GCA AAC
61     K   L   Q   L   Q   A   E   E   R   G   V   V   S   I   K   G   V   C   A   N
241   CGT TAC CTT GCT ATG AAA GAA GAT GGA AGA TTA CTA GCT TCT AAA TGT GTT ACA GAC GAG
81     R   Y   L   A   M   K   E   D   G   R   L   L   A   S   K   C   V   T   D   E
301   TGT TTC TTT TTT GAA CGA TTG GAG TCT AAT AAC TAC AAT ACT TAC CGG TCA AGG AAA TAC
101    C   F   F   F   E   R   L   E   S   N   N   Y   N   T   Y   R   S   R   K   Y
361   TCC AGT TGG TAT GTG GCA CTG AAA CGA ACT GGG CAG TAT AAA CTT GGA CCC AAA ACA GGA
121    S   S   W   Y   V   A   L   K   R   T   G   Q   Y   K   L   G   P   K   T   G
421     CCT GGG CAG AAA GCT ATA CTT TTT CTT CCA ATG TCT GCT AAG AGC TGA
141     P   G   Q   K   A   I   L   F   L   P   M   S   A   K   S   *
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Figure 1: The nucleotide sequence and deduced amino acid sequence of basic fibroblast growth factor from Chinese Small Tail Han sheep

Expression, purification and activity assay of or-bFGF

A 22~kDa predicted protein was induced in the presence of IPTG(Figure 2), the mature recombinant protein comprised full 18kDa-bFGF. The protein was purified by immobilized metal affinity chromatography (Figure 3A) and recognized by antibodies against human recombinant bFGF on western blots(Figure 3B). These results indicated that a predicted fusion protein of bFGF was produced by *E.coli*. containing recombinant plasmid pET-28a/obFGF. In addition, we also determined whether bFGF fused to His-Tag retained biological activity on proliferation of fibroblasts. It was revealed that or-bFGF stimulated the growth of NIH-3T3 cells (Table 1). We also found that or-bFGF stimulated the growth of NIH-3T3 cells to the same extent as human recombinant bFGF (hr-bFGF).Thus, or-bFGF retains its biological activity when fused to His-Tag, providing a good source for further investigation.

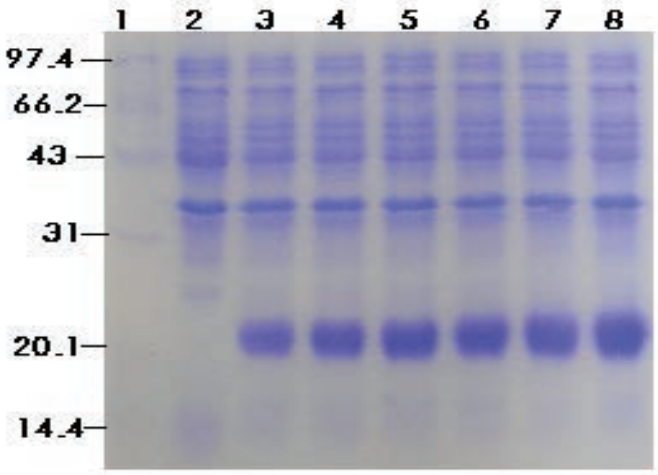

Figure 2: Expression of or-bFGF in *E.coli*. After treating BL21 cells containing pET-28a/bFGF(lane 3~8) or pET-28a(lane 2) with IPTG. Cell lysates were separated on a SDS-PAGE gel

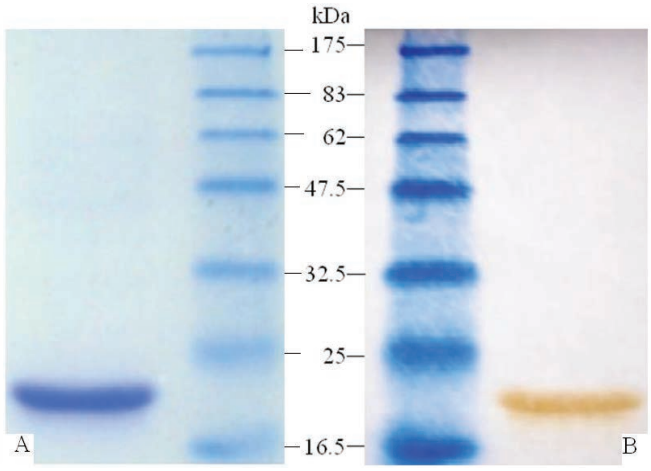

Figure 3: A. Or-bFGF was purified from *E.coli* using Ni-NTA. B. Purified protein was transferred to PVDF membrane and then probed with a monoclonal human bFGF antibody

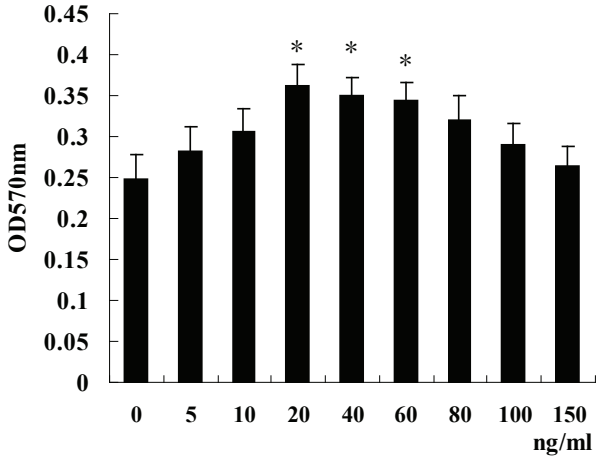

Figure 4: Effects of or-bFGF on proliferation of murine C2C12 myoblasts were detected with MTT assay. bFGF accelerated proliferation of C2C12 cells at the concentrations of 20, 40 and 60ng/ml (*P<0.05), whereas bFGF at other concentrations have no effects (P>0.05)

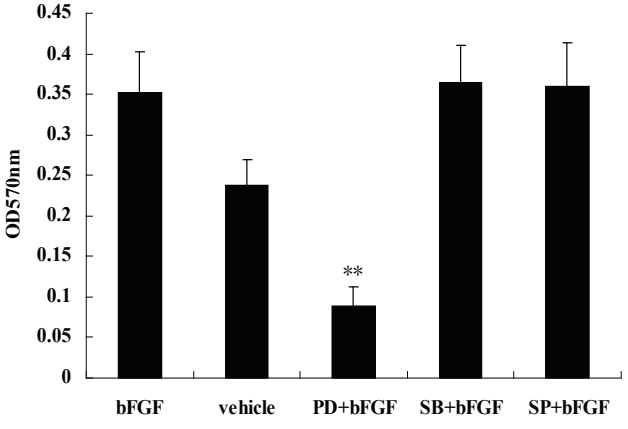

Figure 5: Effects of inhibitors of MAP kinase signaling pathway on proliferation of C2C12 myoblasts induced by bFGF.(PD:PD98059; SB:SB203580; SP:SP600125; **P<0.01, vs bFGF-stimulated cells)

Table 1: The effects of or-bFGF/hr-bFGF on growth of NIH-3T3 fibroblasts

Concentration (ng/ml)	OD570nm	
	or-bFGF	hr-bFGF
0	0.560±0.017	0.566±0.015
5	1.080±0.026	1.083±0.022
10	1.331±0.022	1.351±0.030
20	1.372±0.031	1.359±0.028
50	1.299±0.026	0.311±0.019
80	1.283±0.033	1.272±0.027
100	1.241±0.034	0.223±0.043

Effects of or-bFGF on proliferation of murine C2C12 myoblasts

As shown in Figure 4, the proliferation of murine C2C12 myobalsts were stimulated by or-bFGF significantly ($P<0.05$) at the concentrations of 20, 40 and 60ng/ml but insignificantly ($P>0.05$) at other concentrations. To determine the regulatory mechanisms, three inhibitors were employed respectively to block three MAPK signaling pathways. Results showed that PD98059 significantly suppressed proliferation of myoblasts induced by bFGF($P<0.01$), whereas SP600125 and SB203580 did not affect the proliferation significantly($P>0.05$, Figure 5), which demonstrated that bFGF employs MEK/ERK-MAPK signaling pathway, but not p38-MAPK and SAPK/JNK, to promote proliferation of myoblasts.

Discussion

Molecular characteristics and phylogenetic analysis

On the basis of our investigation, 18kDa-bFGF gene from Chinese Small Tail Han sheep carries most hallmarks attributed to the growth factor, such as receptor-binding sites, RGD sequences, phosphorylation sites and cysteine residues, these characters are important for functions of bFGF, they respectively contribute to binding receptors [20,21], modulation of mitogenicity [22], phosphorylation by protein kinase and forming an intramolecular disulfide bond to stabilize molecular structure [23], so the invariability of these key motifs is conducive to evolutional stabilization of bFGF gene. In addition, results of phylogenetic analysis showed that the growth factor gene remains highly conservation during evolution, the similarities of DNA or protein sequence between Small Tail Han sheep and other published terrestrial animals were found to be high, more than 84.8%, although the similarities between Small Tail Han sheep and amphibian or aquatic animals gradually decrease, but the similarities still keep a high percentage. By this token, bFGF is an evolutional conservative molecule and maybe fit to be regarded as a referenced molecule for biogeny.

Expression and activity assay of or-bFGF

Basic FGF is a growth factor that can stimulate proliferation of murine 3T3 fibroblasts. In this investigation, NIH-3T3 fibroblasts were used to determine biological activity of or-bFGF. In prokaryotic expression system, due to abundant expression, expressional products of exogenous gene probably form inclusion body to lose bioactivity, it is difficult to dissolve and renature the inactive protein. Therefore, to avoid plentiful inclusion body, appropriate conditions were selected and abundant soluble fusion protein was harvested. In addition, for fusion expression system, additional amino acid sequence fused to bFGF maybe affect activity of the target protein. So we detected activity of fusion pritein or-bFGF with NIH-3T3 cells. Results showed that His-Tag did not bring negative effects to bioactivity of the bFGF.

Roles of MEK/ERK-MAPK signaling pathway in proliferation of C2C12 myoblasts induced by bFGF

Basic FGF may be involved in skeletal muscle growth and differentiation by activating signaling pathways independent of PDGF-signaling pathways [24]. Skeletal muscle development and regeneration during embryonic and adult life consisting of proliferation, migration and differentiation of myogenic cells are regulated by several growth factors, in which bFGF has been confirmed to be an important growth factor for skeletal muscle regeneration [13,14,17,25]. Shishkin et al [26] reported that bFGF had no effects on proliferation of myoblasts at the concentrations of 20~80 ng/ml. However, in our experiments, or-bFGF at the concentration of 20~60ng/ml could significantly stimulate the cell proliferation, and or-bFGF at the other concentrations failed to affect proliferation of murine C2C12 myoblasts. As to the proliferation suppression of C2C12 myoblasts by high levels bFGF, it may result from that expression of fibroblast growth factor receptor was impaired by high concentration of exogenous bFGF.

Background researches have shown that bFGF employed different member/members of MAPK family to perform its biological roles in different cells[27-30]. As to skeletal muscle,

it is not clear that which member participates in the regulative mechanism. So we employed three inhibitors respectively to block three MAPK signaling pathways. Results showed that PD98059, inhibitor of MEK/ERK-MAPK signaling pathway, significantly suppressed proliferation of myoblasts induced by bFGF, whereas both of SP600125, inhibitor of SAPK/JNK-MAPK signaling pathway, and SB203580, inhibitor of p38-MAPK signaling pathway, did not affect the proliferation significantly, which demonstrated that bFGF employs MEK/ERK-MAPK signaling pathways, but not p38-MAPK and SAPK/JNK, to promote proliferation of myoblasts.

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References

1. Rosanna D. Fibroblast growth factors as regulators of central nervous system development and function. Am J Physiol Regul Integr Comp Physiol 2003; 284: 868–81.

2. Prats H, Kaghad M, Prats AC. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc Nat Acad Sci U S A 1989; 86:1836–40.

3. Arnaud E, Touriol C, Gersac MC. A new 34-kilo Dalton isoform of fibroblat growth factor 2 is cap dependently synthesized by a survival factor. Mol Cell Biol 1999; 191: 505–14.

4. Andreas B, Sharon K, Giuseppe P. Biological roles of fibroblast growth factor-2. Endocr Rev 1997; 18: 26–45.

5. Kazuho ABE, Hiroshi S. Effects of basic fibroblast growth factor on central nervous system functions. Pharmacol Res 2001; 43: 307–12.

6. Martin P. Wound healing: Aiming for perfect skin regeneration. Science 1997; 276: 75–81.

7. Baird A, Esch F, Mormede P, et al. Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. Recent Prog Horm Res 1986; 42: 143–205.

8. Gospodarowicz D, Ferrara N, Schweigerer L, et al. Structural characterization and biological functions of fibroblast growth factor. Endocr Rev 1987; 8: 95–114.

9. Lobb RR. Clinical applications of heparin-binding growth factors. Eur J Clin Invest 1988; 18: 321–36.

10. Van Neck JW, Medina JJ, Onnekink C, et al. Expression of basic fibroblast growth factor and fibroblast growth factor receptor genes in cultured rat aortic smooth muscle cells. Biochem Biophys Acta 1995; 1261: 210–14.

11. Garrett KL, Anderson JE. Colocalization of bFGF and the myogenic regulatory gene myogenin in dystrophic mdx muscle precursors and young myotubes in vivo. Dev Biol 1995; 169: 596–608.

12. Sharma M, Kambadur R, Matthews KG, et al. Myostatin, a transforming growth factor-β superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. J Cell Physiol 1999;180: 1–9.

13. Lefaucheur JP, Sebille A. Basic fibroblast growth factor promotes in vivo muscle regeneration in murine muscular dystrophy. Neurosci Lett 1995; 202: 121–4.

14. Lefaucheur JP, Sebille A. Muscle regeneration following injury can be modified in vivo by immune neutralization of basic fibroblast growth factor,transforming growth factorβ1 or insulin-like growth factor I. J Neuroimmunol 1995; 57: 85–91.

15. Fu XB, Cuevas P, Gimenez-Gallego G, et al. Ischemia and reperfusion reduce the endogenous basic fibroblast growth factor in rat skeletal muscles: an immunohisto-chemical study. Wound Repair Regen 1996; 4: 381–5.

16. Fu XB, Yang YH, Li XK, et al. Ischemia and reperfusion impair the gene expression of endogenous basic fibroblast growth factor (bFGF) in rat skeletal muscles. J Surg Res 1998; 80: 88–93.

17. Anderson JE, Mitchell CM, Mcgeachie JK, et al. The time course of basic fibroblast growth factor expression in crush-injured skeletal muscle of SJL/J and BALB/c mice. Exp Cell Res 1995; 216: 32–34.

18. Liu HZ, Li Q, Yang XY, et al. Expression of basic fibroblast growth factor results in a decrease of myostatin mRNA in murine C2C12 myoblasts. Acta Biochem Biophys Sin 2006;10:697–703.

19. Liu HZ, An XR, Chen YF, et al. Roles of extracellular signal-regulated kinase 1/2 on the suppression of myostatin gene expression induced by basic fibroblast growth factor in cultured

murine C2C12 myoblasts. Acta Biochem Biophys Sin 2008;11:943-8.

20. Baird A, Schubert D, Lin N. Receptor-and heparin-binding domains of basic fibroblast growth factor. Proc Natl Acad Sci USA 1988;85:2324-8.

21. Nishikage S, Koyama H, Miyata T, et al. In vivo electroporation enhances plasmid-based gene transfer of basic fibroblast growth factor for the treatment of ischemic limb. J Surg Res 2004;120: 37–46.

22. Yayon A, Aviezer D, Safran M. Isolation of peptides that inhibit binding of basic fibroblast growth factor to its receptor from a random phage epitope library. Proc Natl Acad Sci U S A 1993; 90:10643–-7.

23. Presta M, Rusnati M, Urbinati C. Biologically active synthetic fragments of human basic fibroblast growth factor(bFGF): identification of two Asp-Gly-Arg-containing domains involved in the mitogenic activity of bFGF in endothelial cells. J Cell Physiol 1991; 149: 512-24.

24. Templeton TJ, Hauschka SD. FGF-mediated aspects of skeletal muscle growth and differentiation are controlled by a high affinity receptor FGF-R1. Dev Biol 1992; 154: 169–81.

25. Kundla AJ, John ML, Bowen-Pope DF, et al. A requirement for fobroblast growth factor in regulation of skeletal muscle differentiation cannot be replaced by activation of platelet-derived growth factor signaling pathways. Mol Cell Biol 1995; 15: 3238–46.

26. Shishkin SS, Krokhina TB, Akhunov VS, et al. Effects of myostatin and growth factors on cultured human cells. Appl Biochem Microbiol 2004; 40: 547–50.

27. Pages G, Lenormand P, Allemain GL, et al. Mitogen- activating protein kinases p42MAPK and p44MAPK are required for fibroblast proliferation. Proc Natl Acad Sci U S A 1993; 90: 8319–23.

28. Gardner AM, Johnson GL. Fibroblast growth factor-2 suppression of tumor necrosis factor -mediated apoptosis requires Ras and the activation of mitogen-activated kinase. J Biol Chem 1996; 271:14560–6.

29. Tokuda H, Hirade K, Wang X, et al. Involvement of SAPK/JNK in basic fibroblast growth factor-induced vascular endothelial growth factor release in osteblasts. J Endocrinol 2003; 177:101–7.

30. Huang HM, Chang TW, Liu JC. Basic fibroblast growth factor antagonizes activin A-mediated growth inhibition and hemoglobin synthesis in K562 cells by activating ERK1/2 and deactivateing p38 MAP kinase. Biochem Biophys Res Commun 2004; 320: 1247–52.

KLONIRANJE BAZIČNEGA FIBROBLASTNEGA RASTNEGA DEJAVNIKA IZ KITAJSKE KRATKOREPE OVCE PASME HAN IN NJEGOVI UČINKI NA RAZMNOŽEVANJE MIŠJIH MIŠIČNIH CELIC C2C12

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Summary: Za boljše razumevanje lastnosti bazičnega fibroblastnega rastnega dejavnika (bFGF, FGF-2) in njegovih učinkov na skeletne mišice smo klonirali kodirajoče zaporedje 18kDa velikega gena bFGF iz kitajske kratkorepe ovce pasme han, odlične avtohtone pasme na Kitajskem. Zaporedje DNK in prevedena proteinska sekvenca sta bili analizirani in vzporejeni s homologi drugih živalskih vrst. Rezultati so pokazali, da je novo klonirani gen *bFGF* zelo podoben ostalim objavljenim zaporedjem bFGF pri drugih živalskih vrstah, kar nakazuje veliko evolucijsko ohranjenost gena bFGF. Popolno kodirajoče zaporedje gena *bFGF* je bilo vstavljeno v ekspresijski plazmid PET-28a iz česar je nastal fuzijski protein, izražen v *E. coli*. Rekombinantni protein je bil topen in biološko aktiven, primeren za nadaljnjo karakterizacijo. Preučili smo njegov vpliv na razmnoževanje mišjih mišičnih celic C2C12. Ugotovili smo, da ovčji rekombinantni bFGF pospeši njihovo razmnoževanje preko aktivacije signalne poti MEK/ERK-MAPK.

Ključne besede: bFGF; kloniranje; C2C12, MEK / ERK; kratkorepe ovce pasme han

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Original Scientific Article

BACTERIOLOGICAL QUALITY AND SAFETY OF RAW COW'S MILK AND FRESH CREAM

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Summary: In this study, the bacteriological quality and the presence of *S. aureus* and *E. coli* O157 in raw milk and fresh cream were studied. A total of 80 samples (38 raw milk, 38 fresh cream and 4 separator samples) were collected from June to August 2011 at a milk separation center. Samples were analyzed for Standard Plate Count (SPC), total coliforms, faecal coliforms, *E. coli* and *S. aureus* counts as well as for the pathogen *E. coli* O157. Means of counts per ml of milk for SPC, total coliforms, faecal coliforms, *E. coli* and *S. aureus* were 3.62×10^7, 1.65×10^6, 3.69×10^5, 2.83×10^4 and 4.68×10^3 cfu and per ml of cream were 7.79×10^7, 4.21×10^6, 2.07×10^6, 1.89×10^5 and 3.5×10^4 cfu, respectively. *E. coli* and *S. aureus* were isolated from 52.6 and 23.7% of raw milk samples, 47.4 and 31.6 % of fresh cream samples and 75 and 25 % of separators samples, respectively. *E.coli* O157 was detected in 2.6% of raw milk samples. The mean values of titratable acidity for raw milk, fresh cream and separators samples were 0.18, 0.20 and 0.24 %, respectively. Separators play a major role in the increase of the cream microbiota. High microbial counts of both raw milk and fresh cream may present a public health hazard to the consumers and emphasizes the need for improved hygienic standards.

Key words: milk; cream; *S. aureus*; *E. coli* O157; separators

Introduction

Milk is a highly nutritious food that serves as an excellent growth medium for a wide range of microorganisms. Fresh milk drawn from a healthy cow normally contains a low microbial load (less than 1000 cfu/ml milk) but the load may increase up to 100 times fold, or more, once it is stored for sometime at normal temperature (1). Bacteria in raw milk can occur through colonization of the teat canal or an infected udder (clinical and subclinical mastitis) or milk can get contaminated by the surface of the teats, air, milker (manual milking), water and milk contact surfaces, storage and transport equipment (2, 3).

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In Egypt, direct consumption of raw milk is much frequent and more popular than consumption of pasteurized milk because it is believed, especially in rural areas, that raw milk and its byproducts have nutritional advantages over the pasteurized one. However, consumption of raw milk and its byproducts is considered potentially hazardous and has been associated with several types of infections including brucellosis, tuberculosis, salmonellosis, yersiniosis, Escherichia coli O157 and Staphylococcal enterotoxin poisoning (4).

The main producers of milk in Beni-Suef governorate are small farmers with between one and four heads of cattle. There are only a few organized modern and large scale farms. Most farmers milk their cows manually and separate milk into cream and skimmed milk. Raw skimmed milk is used to manufacture Kareish cheese, while

fresh cream is stored at room temperature in a one piece goat skin bag to make butter. Both are sold in a market-place held once a week in each village.

Raw milk (or cream) is the basic material from which all dairy products are made. The diversity of microorganisms and the level of contamination in the raw material has a decisive effect on the quality and safety of the final product. Several studies have been carried out in Egypt to evaluate the bacteriological quality and safety of raw milk and fresh cream (5-10).

Since recent information concerning the bacteriological quality and safety of raw milk and fresh cream in Beni-Suef governorate is sketchy or totally absent, this study was carried out to investigate the bacteriological quality and safety of locally produced raw milk and fresh cream in Beni-Suef governorate.

Material and methods

I. Collections of Samples

The study was conducted in a village milk separation center (containing 4 hand operated separators) in Beni-Suef governorate, Egypt, where milk is manually separated into cream and skimmed milk. Everyday milk separation is performed at room temperature (20- 25 °C), between 6:00 and 10:00 a.m.

A total of 80 samples (38 raw milk, 38 fresh cream and 4 separators samples) was collected from June to August 2011 at the milk separation center. After agitation, milk and cream samples (150 ml) were taken aseptically from farmers containers and stored in sterile screw bottles. After agitation, separators samples (100ml) were obtained from separator bowl "remnants of milk, skim milk and cream", and collected in sterile screw bottles. Samples were transported to the laboratory in an insulated ice box (4 - 6 °C) within 1-2 h of collection and analyzed immediately upon arrival.

II. Bacteriological Analysis

(a) Preparation of Samples: Samples were diluted in 0.1% peptone water (Oxoid, UK) (11 mL of samples in 99 mL of 0.1% peptone water for initial dilution), subsequent decimal dilutions up to 10^7 were prepared with the same diluent and appropriate dilutions were used to enumerate the different groups of microorganisms.

(b) Standard Plate Counts (SPC): SPC was carried out using plate count agar (Oxoid, UK) for 48 ± 3 h after incubation at 32 ± 1 °C (11).

(c) Coliforms, Faecal Coliforms and Escherichia coli: Counting was estimated by a three tube Most Probable Number (MPN) technique (12).

(d) Enumeration, Isolation and Identification of *S. aureus*: *S. aureus* were enumerated by surface spread technique onto Baird Parker agar (Oxoid, UK) (13).

(e) Isolation and Identification of Escherichia coli O157: Twenty five milliliters of each sample was added to 225 mL of modified tryptone soya broth (mTSB) containing 30 g of TSB (Oxoid, UK), 1.5 g of bile salts no.3 (Oxoid, UK), 1.5 g of dipotassium phosphate, and 20 mg of novobiocin (Sigma Chemical Co., St. Louis, MO, USA) per liter. The inoculated broth was incubated at 41.5 $\pm$ 1°C for 18 - 24 h. After 6 h and 18 - 24 h, a loopful of the incubated broth was plated on CT-SMAC agar: Sorbitol Mac Conkey agar (SMAC; Oxoid) supplemented with cefixime and potassium tellurite (0.05 and 2.5 mg/L, respectively; CT supplement, Oxoid). After 18 - 24 h of incubation at 37 ± 1°C, non sorbitol fermenting colonies were selected and isolated. Presumptive colonies of Escherichia coli O157 were biochemically identified using API 20E (Bio Merieux, France). All biochemically identified non sorbitol fermenting colonies were subjected to slide agglutination with E. coli O157 latex test kit (Oxoid) (14).

III. Chemical Analysis

Titratable acidity (TA) (as lactic acid %) of raw milk and fresh cream was measured following the description by O'Connor (15).

IV. Statistical Analysis

SPSS pocket program for windows (version 16, 2007) was used for the statistical analysis. Paired samples T test was used for comparison of means. Values of different parameters were expressed as the mean ± standard error (SE).

Results

Table 1: Bacterial loads of raw milk and fresh cream samples (cfu/mL)

	Raw milk			Fresh cream		
	Min.	Max.	Mean ± SE	Min.	Max.	Mean± SE
SPC*	2.1×10^4	4×10^8	$3.62 \times 10^{7a} \pm 1.37 \times 10^7$	1.5×10^4	7.3×10^8	$7.79 \times 10^{7a} \pm 2.80 \times 10^7$
Total coliforms	< 3	1.5×10^7	$1.65 \times 10^{6\,b} \pm 6 \times 10^5$	< 3	2.4×10^7	$4.21 \times 10^{6\,b} \pm 9.82 \times 10^5$
Faecal coliforms	< 3	7.5×10^6	$3.69 \times 10^{5\,b} \pm 2 \times 10^5$	< 3	2.4×10^7	$2.07 \times 10^{6\,b} \pm 7.64 \times 10^5$
E. coli	< 3	2.4×10^5	$2.83 \times 10^{4\,a} \pm 9.33 \times 10^3$	< 3	2.4×10^6	$1.89 \times 10^{5\,a} \pm 8.89 \times 10^4$
S. aureus	< 10	7×10^4	$4.68 \times 10^{3\,a} \pm 2.44 \times 10^3$	< 10	4×10^5	$3.5 \times 10^{4\,a} \pm 1.7 \times 10^4$

*SPC - standard plate count, [a] means p > 0.05, [b] means p < 0.05

Table 2: Bacterial loads of separators samples (cfu/mL)

	Min.	Max.	Mean	± SE.
Spc*	3.1×10^7	3.5×10^8	1.88×10^8	6.77×10^7
Total coliforms	2.4×10^7	$> 1.1 \times 10^8$	8.85×10^7	2.15×10^7
Faecal coliforms	7.5×10^5	9.3×10^6	3.46×10^6	1.97×10^6
E. coli	< 3	2.1×10^5	1.06×10^5	6×10^4
S. aureus	< 10	1.6×10^4	4×10^3	4×10^3

*SPC - standard plate count

Table 3: Incidence of pathogens in examined samples

	No of samples	*E. coli*		*S. aureus*		*E. coli* O157	
		No	%	No	%	No	%
Milk	38	20	52.6	9	23.7	1	2.6
Cream	38	18	47.4	12	31.6	0	0
Separators	4	3	75	1	25	0	0

Table 4: Results of Titratable acidity of examined samples

	Minimum	Maximum	Mean ± SE
Raw milk	0.13	0.30	0.18 ± 0.01 [a]
Fresh cream	0.13	0.32	0.20 ± 0.01 [a]
Separators	0.20	0.31	0.24 ± 0.03

a means p > 0.05

The incidence of different microorganisms in raw milk and fresh cream is shown in Table 1. The SPC of raw milk and fresh cream ranged from 2.1 x 10^4 - 4 x 10^8 and 1.5 x 10^4 - 7.3 x 10^8 cfu/ml with a mean count of 3.62 x 10^7 ± 1.37 x 10^7 and 7.79 x 10^7 ± 2.8 x 10^7 cfu/ ml, respectively. Only 2.6 and 5.3 % of the examined raw milk and fresh cream samples were found to be in accordance with the <1 x 10^5 and < 3 x 10^4 cfu/ml set by Robinson (2002) for raw milk intended for further processing and fresh cream, respectively (Figure 1). Total coliforms and faecal coliforms were detected in 89.5 and 65.8 % of examined raw milk samples with a mean value of 1.65 x 10^6 ± 6 x10^5 and 3.69 x 10^5 ± 2 x10^5 MPN/ml and in 94.7 and 78.9 % of the examined fresh cream samples with a mean value of 4.21 x 10^6 ± 9.82 x 10^5 and 2.07 x 10^6 ± 7.64 x 10^5 MPN/ml, respectively (Table 1 and Figure 2, 3). The mean values for separators samples were 1.88 x 10^8 ± 6.77x 10^7 for SPC, 8.85 x 10^7 ± 2.15 × 10^7 for total coliforms, 3.46 x 10^6 ± 1.97 x 10^6 for faecal coliforms, 1.06 ×10^5 ± 6 x10^4 for *E. coli* and 4 x10^3 ± 4 ×10^3 cfu/ml for *S. aureus* (Table 2).

E. coli was isolated from 20 (52.6%) of 38 raw milk and 18 (47.3%) of 38 fresh cream samples with a mean count of 2.83 x 10^4 ± 9.33 x 10^3 and 1.89 x 10^5 ± 8.89x 10^4 cfu/ml, respectively . Nine (23.7%) of 38 raw milk samples and 12 (31.6%) of 38 fresh cream samples were contaminated with *S. aureus*, with an average of 4.68 x 10^3 ± 2.44 x 10^3 and 3.5 x 10^4 ± 1.7 x 10^4cfu/ml, respectively (Table 1, 3). *E. coli* and *S. aureus* were isolated form 75 and 25 % of the examined separators samples, respectively. E.coli O157 was isolated from one (2.6%) of 38 raw milk samples. None of the fresh cream and separators samples taken contained detectable levels of *E. coli* O157 (Table 3). Mean values of titratable acidity for raw milk, fresh cream and separators samples were 0. 18 ± 0.01, 0.20 ± 0.01 and 0.24 ± 0.03%, respectively (Table 4).

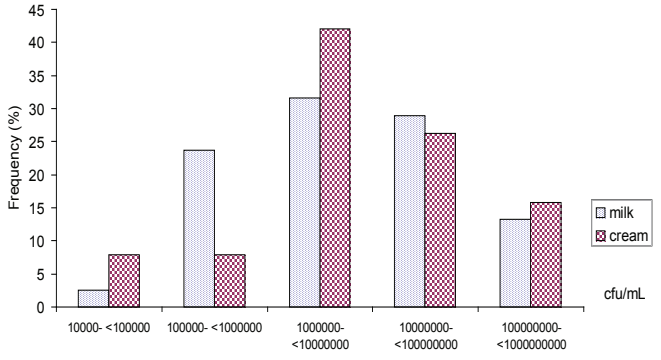

Figure 1: Frequency distribution of SPC in milk and cream samples

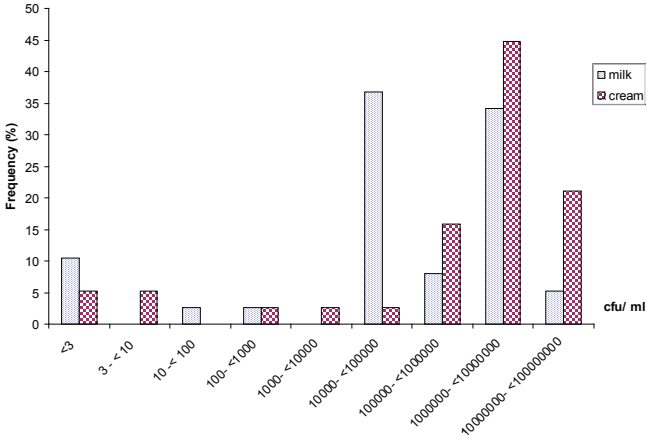

Figure 2: Frequency distribution of coliforms in milk and cream samples

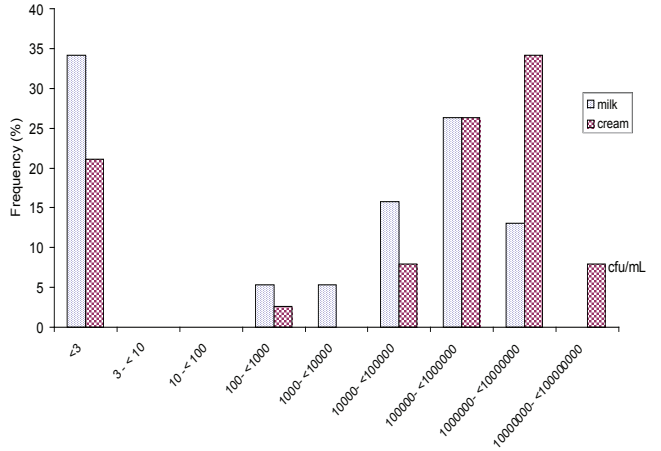

Figure 3: Frequency distribution of faecal coliforms in milk and cream samples

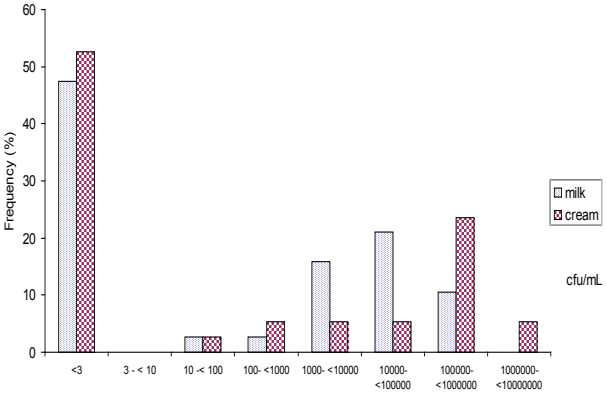

Figure 4: Frequency distribution of *E. coli* in milk and cream samples

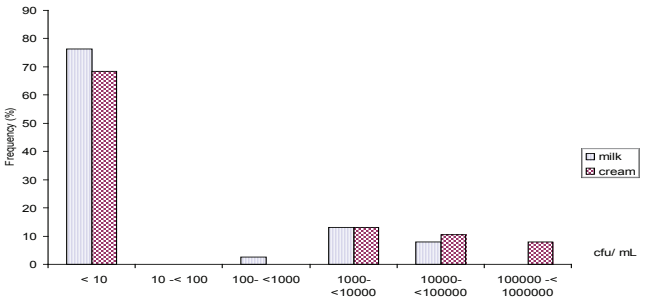

Figure 5: Frequency distribution of *S. aureus* in milk and cream samples

Discussion

The bacterial count in milk and cream potentially reveals the general conditions of sanitation and temperature control under which milk and cream were produced, handled and held.

Standard Plate Counts (SPC)

Raw milk contained an average SPC of 3.62 x 10^7 ± 1.37 x 10^7 cfu/ ml. Al-Tarazi et al. (17) and Korashy and Mohamed (9) reported considerably lower levels of SPC in raw milk with mean values of 1.1 x 10^7 and 4 x 10^6 cfu/ml, respectively. On the contrary, high levels of SPC in raw milk were reported by Moustafa et al. (5) and Sobeih et al (8) with mean values of 4 X 10^7 and 2.9 X 10^8 cfu/mL, respectively. The results found in those studies and present work indicate that raw milk and fresh cream are heavily contaminated. The mean count of SPC of fresh cream was marginally higher than those of raw milk but statistically not significant (P > 0.05). Possible reasons for the high counts could be due to infected udders of the cows, lack of knowledge about clean milk production, use of unclean equipment, poor personal hygiene, lack of cooling after milking and lack of heat treatment, which contribute to the poor hygienic quality of raw milk and fresh cream. Therefore, training and guidance should be given to the farmers in general milking hygienic practices and in keeping milk at low temperature to avoid microbial growth and lengthen the shelf life.

Coliforms, Faecal Coliforms and Escherichia coli

Many reports dealing with the occurrence of coliforms in raw milk have been accumulated. In those studies, various rates of coliforms were reported as 100, 100, 96, 88.7, 90, 41.3, 80 and 100% of examined raw milk samples by Saudi and Moawad (6), Ahmed and Sallam (18), Sobeih et al.(8), Al-Tarazi et al. (17), Chye et al. (19) , Korashy and Mohamed (9), Altalhi and Hassan (2) and El-Prince et al. (10), respectively. El-Essawy and Riad (20) and El-kosi (7) reported that all (100%) examined fresh cream samples were contaminated with coliforms. There are several reasons for these variations, such as differences in hygienic practices during milking, differences in geographic location and differences in seasonal trends. According to Robinson (16), total coliforms of raw milk intended for further processing should be < 500 cfu/mL and for fresh cream < 30 cfu/ mL. Thirty-two (84.2%) milk samples tested and 34 (89.5%) cream samples tested were found to be highly contaminated with coliforms over this limit (Figure 2).

The existence of coliforms may not necessarily indicate a direct faecal contamination of milk and cream, but is an indicator of poor hygiene and sanitary practices during milking and further handling, and presents potential hazard for people consuming such products. A significant difference occurred between the total coliforms of milk and total coliforms of cream (P< 0.05), suggesting that allowing milk samples temperature to resemble environmental temperature will favour the growth of different types of bacteria and could be responsible for high coliforms count in fresh cream.

E. coli and coliforms are often used as indicator microorganisms, and the presence of *E. coli* implies a risk that other enteric pathogens may be present in the sample. *E. coli* was isolated from 20 (52.6%) milk samples and 18 (47.3%) fresh cream samples (Table 3). All positive raw milk and fresh cream samples do not comply with Robinson (16) standards of ≤ 1 cfu *E. coli*/mL (Figure 4). The contamination rate in raw milk samples was extremely lower than the findings of Moustafa et al. (5), Sobeih et al. (8), Soomro et al. (21), Chye et al (19) and Altalhi and Hassan (2) as they found 66.6, 88, 65, 65 and 66% of their samples were contaminated by *E. coli*, respectively, but higher than the rate of 32, 27.5 and 3.3% reported by Ahmed and Sallam (22), Mezyed et al.(23) and El-Prince et al. (10), respectively. In previous studies, Ahmed and Sallam (22) and Mezyed et al. (23) reported that 38 and 15% of the cream samples tested were contaminated by *E. coli*, respectively.

Detection of *E. coli* in milk often reflects faecal contamination, although environmental coliforms have also been detected in milk. Milk can be easily contaminated by infected food handlers who practice poor personal hygiene or by water containing human discharges. Therefore, farmers must be educated in safe handling techniques and proper personal hygiene practices, including hand washing. Using potable water in dairy farm operations is crucial. Water must be safe and practically free from any type of bacterial contamination that may affect milk quality (19).

Staphylococcus aureus in raw milk and fresh cream

In the present study, 9 (23.7%) raw milk samples and 12 (31.6%) fresh cream samples were contaminated with *S. aureus* (Table 3). All positive samples were above the limits (100 cfu/mL) established by Robinson (16) standards (Figure 5). The isolation rate observed in this study was similar to those reported by Abdel-Hameed and El-Malt (24) who reported that 24 % of the examined milk samples were contaminated with *S. aureus*. However, Al-Tarazi et al. (17), El-Ziney and Al-Turki (25) and Guven et al. (26) reported higher levels of contamination for milk as they found 47, 70 and 33.3% of the milk samples were contaminated with *S. aureus*. *S. aureus* is frequently found in raw milk and milk products. Infections of the mammary gland (mastitis) represent a significant reservoir of toxigenic strains in raw milk. Storage of raw milk before separation under high environmental temperature permitting growth of *S. aureus* can stimulate the production of *S. aureus* enterotoxin. A significant difference did not occur between *S. aureus* count in milk and in cream ($P > 0.05$). This result highlights the unhygienic handling and inadequate personal hygiene.

S. aureus is one of the most common causes of food poisoning in humans worldwide. Although all raw milk and fresh cream samples have lower counts of *S. aureus* than 10^6 - 10^8 cfu/mL levels that are regarded as significant for human food poisoning to occur (27, 28**)**, they still present a public health hazard. Therefore, general hygienic practices aimed at minimizing bacterial contamination of milk and cream should be emphasized, as well as the growth of *S. aureus* must be prevented to avoid potential risk. Neither the absence of *S. aureus* nor the presence of small numbers of organism can provide complete assurance that the milk and cream are safe, since conditions inimical to the survival of *S. aureus* may result in a diminished population or death of viable microbial cells, while sufficient toxins remain to elicit symptoms of staphylococcal food poisoning (29).

Escherichia coli O157

E. coli O157 was isolated from one (2.6%) raw milk sample. None of the fresh cream and separators samples taken contained detectable levels of *E. coli* O157 (Table 3). There are a number of studies from Egypt and different countries concerning the incidence of *E. coli* O157 on raw milk and cream. Abdul-Raouf et al. (30), Abdel Khalek et al. (31) and Amer and Soliman (32) reported 6, 2 and 1% of raw milk examined in Egypt were contaminated with *E. coli* O157:H7, respectively. Allerberger and Dierich (33) reported 3% of the milk samples tested in Austria to be positive for *E. coli* O157:H7, Klie et al. (34) found that 3.9% of the raw milk analyzed in Germany was contaminated with *E. coli* O157:H7 and Chye et al. (19) detected *E. coli* O157:H7 in 33.5% of raw milk samples in Malaysia.

Although *E. coli* O157 was not detected in the cream and separators samples, the presence of other *E. coli* indicated that the potential modes of contamination by pathogenic *E. coli* are present during cream processing and handling. In previous survey, El-Kosi (7) reported that 20% of raw cream samples were contaminated with *E. coli* O157. This value is extremely higher than that obtained from this study.

Although the consumption of undercooked ground beef is still the traditional mode for *E. coli* O157:H7 infections, illnesses resulting from ingestion of contaminated raw milk are increasing. Clinical manifestations range from asymptomatic carriage through mild diarrhea to life-threatening conditions (35). The environmental niches for *E. coli* O157:H7 have not yet been clearly established. However, beef and dairy cattle appear to be a major reservoir for this pathogen (36). *E. coli* O157:H7 is apparently confined to the intestinal tract of dairy cattle and perhaps other animals as well. Therefore, preventing faecal material from contaminating the milk is an important step in reducing the prevalence of *E. coli* O157 and other pathogens in raw milk.

Chemical analysis

The mean values of titratable acidity for raw milk, fresh cream and separators samples were 0.18 ± 0.01, 0.20 ± 0.01 and 0.24 ± 0.03%, respectively (Table 4). Similar TA was reported in earlier studies on raw milk by Al-Zenki et al. (37) and Tasci (38) stating that the mean value of TA was 0.18%. On the other hand, higher values (0.199 and 0.23%) of TA for raw milk were reported by Al-Tarazi et al. (17) and Tassew and Seifu (39), while lower mean values (0.156 and 0.16%) of TA for raw milk were reported by Soler and Ponsell (40) and Korashy and Mohamed (9), respectively.

Fresh milk has a titratable acidity of 0.14 to 0.16% expressed as lactic acid(41) and loses its keeping quality when a critical acidity of 0.200 ± 0.01% is reached (37). However, 25 (65.79%) raw milk samples evaluated during the course of this study did not reach this critical value of acidity, thus still possessing good processing quality according to this quality parameter. A significant difference did not occur between TA of raw milk and fresh cream (P> 0.05). The increased acidity in raw milk and fresh cream may have been caused by keeping the milk at high room temperature (20 - 25°C) that favours the growth of lactic acid forming bacteria and other types of bacteria in the period between milk production and separation.

The use of inadequately cleaned and sanitized milking equipment is considered to be the major source of bacteria found in milk after its collection (42). The growth of a variety of microorganisms is supported by milk residues or remaining washing water left on milking equipment contact surface support. Bacteria multiply within these residues and contaminate milk passing through the equipment (43). In this study, according to the separators owner, separators were washed with water only, which could partly explain the high levels of bacterial contamination. According to the data portrayed in Table 2, it could be concluded that the separators had a definite effect on the cream quality.

Conclusions

Results of the study clearly indicate that bacteriological quality and safety of both raw milk and fresh cream produced by farmers were inferior. Separators are the main source of cream contamination and frequently the principal cause of high bacterial counts. High bacterial counts are likely to affect the keeping quality and safety of raw milk and fresh cream as well as products derived from it. The presence of pathogenic bacteria such *E. coli* O157 and *S. aureus* may pose a risk for public heath. Therefore, it is necessary to develop the hygienic status of locally produced raw milk and fresh cream, through educating the farmers in general hygienic practices and in handling their foods including correct storage to protect them from infection and to save a lot of products from deterioration. Also, information on health hazards associated with consumption of raw unpasteurized milk should be extended to the public, so that consumption of untreated raw milk and its products could be avoided.

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Refferences

1. Richter RL, Ledford RA, Murphy SC. Milk and milk products**.** In: Vanderzant C, Splittstoesser DF, eds. Compendium of methods for the microbiological examination of foods. 3rd ed. Washington: American Public Health Association, 1992: 837–56.

2. Altalhi AD, Hassan SA. Bacterial quality of raw milk investigated by E. coli and isolates analysis for specific virulence -gene markers. Food Control 2009; 20: 913–7.

3. Bytyqi H, Bigler S, Muji S, Jahja A, Zaugg U. Survey on raw milk quality in Kosovo. Food Nutr Sci 2011**;** 2: 414–21.

4. Baylis CL. Raw milk and raw milk cheese as vehicles for infection by verocytotoxin- producing *Escherichia coli*. Int J Dairy Technol 2009; 62: 293–307.

5. Moustafa MK, Ahmed AA H, Abdel-Hakiem EH. Sanitary condition of market milk in Assiut city. Assiut Vet Med J 1988; 19 (38): 80–4.

6. Saudi AM, Moawad AA. Incidence of Enterobacteriaceae in market milk in Cairo and its suburbs. Assiut Vet Med J 1990; 24 (47): 134–9.

7. El-Kosi OHR. Occurrence of some enteric pathogens and their indicators in some Egyptian raw milk products. Assiut Vet Med J 2001; 45: 48–61.

8. Sobeih AMK, Al-Hawary II, Aman IM. Microbiological quality of milk and ice cream sold in Kafr El-Sheikh and El-Gharbia governorates. Minufyia Vet J 2002; 2: 79–88.

9. Korashy E, Mohamed N. Chemical and microbiological analysis to evaluate the sanitary condition of raw milk in Assiut. Assiut Vet Med J 2008; 54(118): 38–50.

10. El-Prince E, Sayed M, Farghaly M, Abdel-Rahman AM. Investigation of milk and some dairy products for fecal pollution indicators. Assiut Vet Med J 2010; 56 (127): 96–107.

11. Laird DT, Gambrel-Lenars SA, Scher FM, Graham TE, Reddy R. Microbiological count methods. In: Wehr HM, Frank JF, eds. Standard methods for the examination of dairy products. 17th ed. Washington: American Public Health Association, 2004: 153–86.

12. Kornacki JL, Johnson JL. Enterobacteriaceae, coliforms and *Escherichia coli* as quality and safety indicators. In: Downes FP, Ito K, eds. Compendium of methods for the microbiological examination of foods. 4th ed. Washington: American Public Health Association, 2001: 69–82.

13. Henning DR, Flowers RS, Reiser R, Ryser ET. Pathogens in milk and milk products. In: Wehr HM, Frank JF, eds. Standard methods for the examination of dairy products. 17th ed. Washington: American Public Health Association, 2004: 103–51.

14. Roberts D, Greenwood M. Practical food microbiology. 3rd ed. London: Blackwell Publishing, 2003.

15. O'Connor CB. Rural dairy technology: ILRI training manual 1. Addis Ababa, Ethiopia: International Livestock Research Institute, 1995.

16. Robinson RK. Dairy microbiology handbook, the microbiology of milk and milk products. 3rd ed. New York: John Wiley and Sons, 2002: 655-736.

17. Al-Tarazi Y, Al-Zamil A, Shaltout F, Abdel-Samei H. Sanitary status of raw cow milk marketed in northern Jordan. Assiut Vet Med J 2003; 49: 180–94.

18. Ahmed AM, Sallam SS. Public health significance of coliforms in raw milk and domiati cheese. Assiut Vet Med J 1991; 25(50): 89–92.

19. Chye FY, Abdullah A, Ayob MK. Bacteriological quality and safety of raw milk in Malaysia. Food Microbiol 2004; 21: 535–41.

20. El-Essawy HA, Riad AAM. Incidence of coliform organisms in cream and ice-cream with special reference to enteropathogenic strains of *Escherichia coli*. Assiut Vet Med J 1990; 24: 140–4.

21. Soomro AH, Arain MA, Khaskheli M, Bhutto B. Isolation of Escherichia coli from raw milk and milk products in relation to public health sold under market conditions at Tandojam. Pak J Nutr 2002; 1: 151–2.

22. Ahmed AM, Sallam SS. Prevalence of *Escherichia coli* serotypes in raw milk and some dairy products. Assiut Vet Med J 1991; 25: 93–7.

23. Mezyed EM, Sharaf EM, Abou El-Roos NA. Occurrence of some enteric pathogens in raw milk and some dairy products. Vet Med J Giza 2008; 56: 29–36.

24. Abdel-Hameed KG, El-Malt LM. Public health hazard of *S. aureus* isolated from raw milk and ice cream in Qena governorate. Assiut Vet Med J 2009; 55: 191–200.

25. El-Ziney MG, Al-Turki AI. Microbiological quality and safety assessment of camel milk (*Camelus dromedaries*) in Saudi Arabia (Qassim region). Appl Ecol Environ Res 2007; 5: 115–22.

26. Guven K, Mutlu MB, Gulbandilar A, Cakir P. Occurrence and characterization of *Staphylococcus aureus* isolated from meat and dairy products consumed in Turkey. J Food Saf 2010; 30: 196–212.

27. Kerouanton A, Hennekinne JA, Letertre C, et al. Characterization of *Staphylococcus aureus* strains associated with food poisoning in France. Int J Food Microbiol 2007; 115: 369–75.

28. Quinn PJ, Carter ME, Markey B, Carter GR. Clinical veterinary microbiology. Edinburgh: Mosby, 2002.

29. Bennett RW, Monday SR. *Staphylococcus aureus*. In: Miliotis MD, Bier JW, eds. International handbook of food borne pathogens. New York: Marcel Dekker, 2003: 240–58.

30. Abdul-Raouf UM, Ammar MS, Beuchat LR. Isolation of *Escherichia coli* O157:H7 form some Egyptian foods. Int J Food Microbiol 1996; 29: 423–6.

31. Abdel Khalek A, El Gaml A, El Sherbini M. Prevalence of *Escherichia coli* O157 in milk and faeces of dairy farm animals in Dakahlia province. In: 1st congress of food hygiene & human health. Assiut, Egypt: Department of Food Hygiene, Faculty of Veterinary Medicine, 2001.

32. Amer AA, Soliman NF. Prevalence of enterohaemorrhagic *Escherichia coli* O157:H7 in raw milk and effect of some chemical preservatives on its viability. Assiut Vet Med J 2004; 50(102): 33–47.

33. Allerberger F, Dierich MP. Enterohaemorrhagic *Escherichia coli* in Austria. In: VTEC 97. 3rd International Symposium and Workshop on Shiga Toxin (Verocytotoxin) - producing *Escherichia coli* infections. Baltimore, 1997. Melville, NY: Lois Joy Galler Foundation for HUS, 1997: abstr. V37/I

34. Klie H, Timm M, Richter H, Gallien P, Perlberg KW, Steinruck H. Detection and occurrence of verotoxin-forming and /or shigatoxin producing *Escherichia coli* (VTEC and /or STEC) in milk. Berl Munch Tierarztl Wochenschr 1997; 110: 337–41.

35. Tarr PI, Gordon CA, Chandler WL. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet 2005; 365(9464): 1073–86.

36. Ryser ET. Public health concerns In: Marth EH, Steele JL, eds. Applied dairy microbiology. 2nd ed. pp. 397-545. New York: Marcel Dekker, 2001: 397–545.

37. Al-Zenki SF, Al-Mazeedi HM, Al-Hooti SN, et al. Quality and safety characteristics of milk sold in the state of Kuwait. J Food Process Preserv 2007; 31: 702–13.

38. Tasci F. Microbiological and chemical properties of raw milk consumed in Burdur. J Anim Vet Adv 2011; 10 : 635–41.

39. Tassew A, Seifu E**.** Microbial quality of raw cow's milk collected from farmers and dairy cooperatives in Bahir Dar Zuria and Mecha district, Ethiopia. Agric Biol J North Am 2011; 2(1): 29–33.

40. Soler A, Ponsell C. The microbiological quality of milk produced in the Balearic Islands. Int Dairy J 1995; 5: 69–74.

41. Fox PF, McSweeney PLH. Physical properties of milk. In: Dairy chemistry and biochemistry. London: Blackie Academic & Professional, 1998: 437–61.

42. Chambers JV. The microbiology of raw milk. In**:** Robinson RK, ed. Dairy microbiology handbook, the microbiology of milk and milk products. 3rd ed. New York: John Wiley and Sons, 2002: 39–90.

43. Hayes MC, Boor K. Raw milk and fluid milk products. In: Marth EH, Steele JL, eds. Applied dairy microbiology. 2nd ed. New York: Marcel Dekker, Inc. USA. 2001: 59–76.

BAKTERIOLOŠKA KAKOVOST IN VARNOST SUROVEGA KRAVJEGA MLEKA IN SVEŽE SMETANE

A. M. S. Meshref

Povzetek: V raziskavi smo preučevali bakteriološko kakovost in prisotnost bakterij *S. aureus* in *E. coli* O157 v surovem mleku in sveži smetani. V zbiralnici mleka smo od junija do avgusta 2011 zbrali 80 vzorcev (38 vzorcev surovega mleka, 38 vzorcev sveže smetane in 4 vzorce iz ločevalnika). V vzorcih smo ugotavljali standardno število mikroorganizmov (SPC), skupne koliformne bakterije in koliformne bakterije fekalnega izvora (*E. coli* in *S. aureus*) ter patogene bakterije *E. coli* seva O157. Povprečne vrednosti CFU na ml mleka za SPC, skupne koliformne bakterije, koliformne bakterije fekalnega izvora, *E. coli* in *S. aureus*, so bile naslednje: 3,62 x 107, 1,65 x 106, 3,69 x 105, 2,83 x 104 in 4,68 x 103; na ml smetane pa: 7,79 x 107, 4,21 x 106, 2,07 x 106, 1,89 x 105 in 3,5 x 104. *E. coli* in *S. aureus* sta bila izolirana iz 52,6 % oziroma 23,7 % vzorcev surovega mleka, iz 47,4 % oziroma 31,6 % vzorcev smetane in iz 75 % oziroma 25 % vzorcev iz ločevalnika. E. coli O157 smo odkrili pri 2,6 % vzorcih surovega mleka. Srednje vrednosti kislosti v vzorcih surovega mleka, sveže smetane in ločevalnih vzorcih so bile 0,18 %, 0,20 % in 0,24 %. Visoke mikrobne vrednosti v vzorcih surovega mleka in sveže smetane lahko predstavljajo tveganje za zdravje potrošnikov in kažejo na nujnost izboljšanja higienskih standardov.

Ključne besede: mleko; smetana; *S. aureus; E. coli* O157; ločevalni vzorci

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Case report

ANOGENITAL CLEFT IN A BITCH – A CASE REPORT

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Summary: A female 4-month-old Pug dog was referred to the Small Animal Clinic of the Faculty of Veterinary Medicine, Trakia University, Stara Zagora with a history of difficult and painful urination, reddening and swelling of external genitalia since one month of age. The owner had noticed the anomaly several days after the birth of the puppy, but had not sought medical advice as there were no signs of discomfort. The physical examination did not reveal any deviations from the normal state. The inspection of external genitalia showed an incomplete occlusion of the skin between the dorsal commissure of the vulva and the anus, exposing directly the swollen and hyperaemic vaginal vestibule and clitoris. The diagnosis of congenital incomplete closure of the anogenital space (anogenital cleft) was made and surgical repair of the anomaly was performed. The anomaly was corrected surgically by creating a dorsal vestibular wall by means of inverted V-shaped perineoplasty. An inverted V-shaped incision was made on the skin between the anus and dorsal commissure of the vulva, followed by removal of the tissue between the vestibular mucosa and the skin edge. The wall of the vaginal vestibule was closed by interrupted absorbable 2-0 polyglycolic acid sutures and the skin – with interrupted non-absorbable sutures. The outcome of the operation was excellent, without any postoperative complications or other genital defects. The follow-up examination performed 5 month later showed a normal position of the external genitalia and the anogenital space.

Key words: bitch; anogenital cleft; perineoplasty

Introduction

Congenital anomalies in the region of the anus, rectum and external genitalia are observed mainly in dogs and cats (1, 2, 3, 4, 5). These pathological conditions are rarely encountered in the clinical practice but nevertheless, they are important as in most cases, they are life-threatening for neonate animals (2, 3).

Congenital anogenital anomalies are mainly seen in dogs and could be present as anal stenosis, atresia ani, anus vestibularis, anogenital cleft, cloaca and rectogenital or rectourethral fistula (2, 4, 5, 6, 7, 8, 9, 10, 11, 12).

The congenital lack of anogenital space is an incomplete closure of the skin fold between the anus and the dorsal commissure of the vulva. This anomaly is called anovulvar (13, 14), anovaginal (15, 16), anogenital (11), vulvovaginal (12) or vulvovestibular cleft (17).

The anogenital cleft is a rare congenital anomaly in dogs and cats due to incomplete closure of dorsal urogenital folds (7, 17, 18). An incomplete occlusion of the skin between the dorsal vulvar commissure and the anus is observed, exposing the bottom of the vaginal vestibule and the clitoris. This defect could be also seen in hermaphroditism (19).

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Case history

A female 4-month-old Pug dog was referred to the Small Animal Clinic of the Faculty of Veterinary Medicine, Trakia University – Stara Zagora with a history of difficult and painful urination, reddening and swelling of external genitalia since one month of age. The owner had noticed the anomaly several days after the birth of the puppy, but had not sought medical advice as there were no signs of discomfort. There were no other littermates with the same disorder, the animal had not suffered from other illnesses and had not been treated on any occasion.

The physical examination did not reveal any deviations from the normal state. The puppy was female, 4-month-old, weighed 6.7 kg, with preserved appetite and normal values of rectal temperature, respiratory and heart rates. The inspection of external genitalia showed an incomplete occlusion of the skin between the dorsal commissure of the vulva and the anus, exposing directly the swollen and hyperaemic vaginal vestibule and clitoris (Fig. 1).

On the basis of summarized data from the history and physical examination, the diagnosis of congenital incomplete closure of the anogenital space (anogenital cleft) was made and surgical repair of the anomaly was performed.

The operation site was aseptically prepared. Premedication was done by subcutaneous injection of 0.04 mg/kg atropine sulfate (Atropinum sulfuricum; Sopharma; Bulgaria). The induction of anaesthesia was done by intravenous application of the combination 0.4 mg/kg diazepam (Diazepam; Sopharma; Bulgaria) and 10 mg/kg ketamine (Ketaminol 10; Intervet; Holland). After endotracheal intubation, inhalation anaesthesia was maintained with isoflurane (Forane; Abbott Laboratories Ltd; United Kingdom). The surgical correction of this congenital defect consisted in inverted V-shape perineoplasty described by Wilson and Clifford (7) and Burt and Smith (20). An inverted V-shaped incision was made on the skin between the anus and dorsal commissure of the vulva, followed by removal of the tissue between the vestibular mucosa and the skin edge. The wall of the vaginal vestibule was closed by interrupted absorbable 2-0 polyglycolic acid sutures (Marlin; Catgut GmbH; Markneukirchen) (Fig. 2) and the skin – with interrupted non-absorbable Vitalon No 0 sutures (Dr Hammer & Co. GmbH, Hamburg) (Fig. 3).

Post operative treatment consisted in intramuscular administration of 30 mg/kg Lincomycin/Spectinomycin 5/10 (Alfasan International; Holland) for 5 days and 2 mg/kg ketoprofen (Ketofen; Merial; Lion; France)

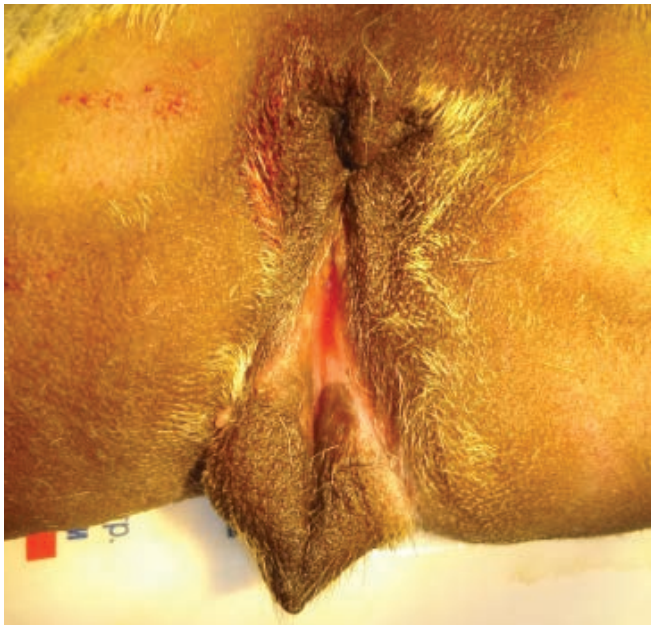

Figure 1: Appearance of the patient by the time of its referral at the clinic – incomplete closure of the anogenital space, hyperaemia and tissue swelling

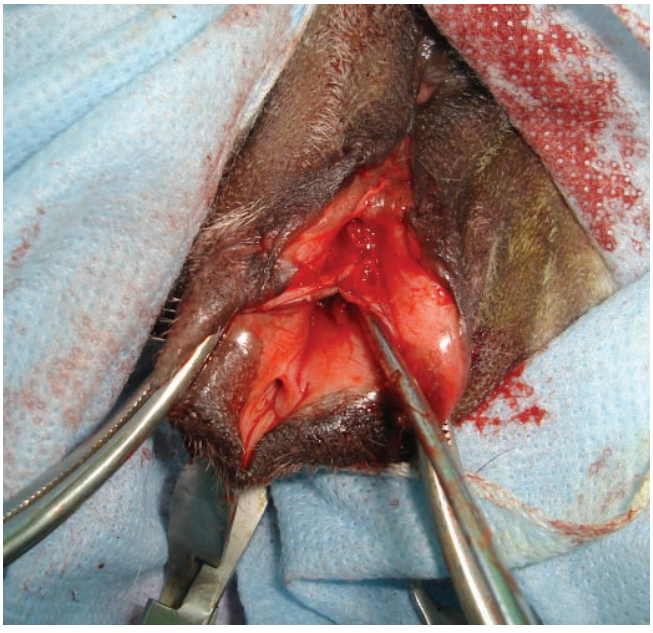

Figure 2: Closure of the vaginal vestibular wall by simple interrupted sutures

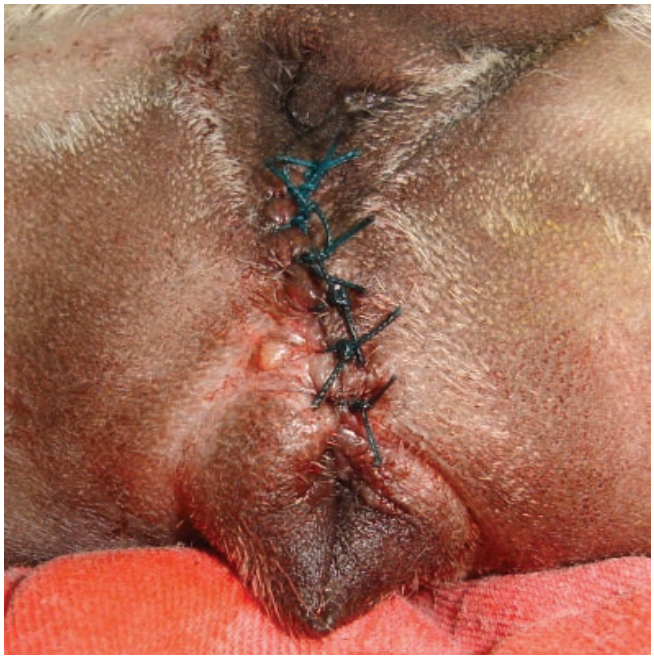

Figure 3: Final appearance of the anogenital space after the surgical correction

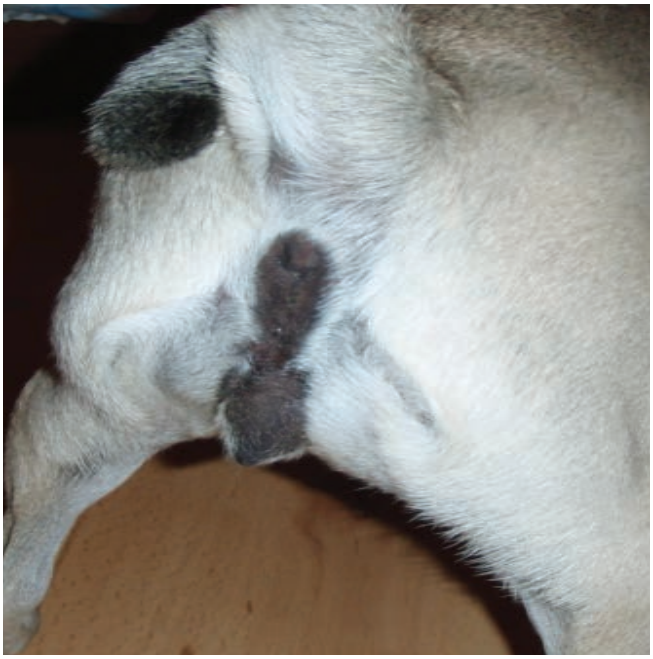

Figure 4: Patient's appearance 5 months after the surgery – normal position of external genitalia and of anogenital space

for 3 days. A protective Elizabethan collar was placed. Skin sutures were removed by the 10th day after the defect repair and by that time, the recovery was evaluated as good. The follow-up examination performed 5 months later showed a normal position of the external genitalia and the anogenital space (Fig. 4).

Discussion

This case report described a rare congenital anogenital anomaly in the dog.

According to White (19), the congenital incomplete occlusion of the skin between the anus and dorsal vulvar commissure is related to staining of the clitoris and the vaginal vestibule with faeces and exposure of these anatomical structures to external influences. Thus, this defect requires a surgical repair by the inverted V-shaped perineoplasty technique (7, 18, 19, 20).

Another possible complication of this defect is vaginal hyperplasia (13) and that is why we performed the surgical correction before the first oestrus of the patient.

In this puppy, the operative intervention using inverted V-shaped perineoplasty gave an excellent result, was performed easily and without post operative complications.

The choice of this technique for correction of the described defect relied upon the expected good effect consisting of prevention of contamination and infection of external genitalia and the excellent cosmetic effect (17).

The prognosis for the congenital incomplete occlusion of the anogenital space is good when treated surgically and timely similarly to reports of other authors (7, 13, 20).

References

1. Niebauer GW. Rectum, anus, and perianal and perineal regions. In: Harvey CE, Newton CD, Schwartz A, eds. Small animal surgery. Philadelphia: J. B. Lippincott, 1990: 381–402.

2. Matthiesen DT, Marretta SM. Diseases of the anus and rectum. In: Slatter D, ed. Textbook of small animal surgery. 2nd ed. Philadelphia: W. B. Saunders, 1993: 627–45.

3. Niebauer GW. Rectoanal disease. In: Bojrab MJ, ed. Disease mechanisms in small animal surgery. 2nd ed. Philadelphia: Lea & Febiger, 1993: 271–84.

4. Hoskins JD, Dimski DS. The digestive system. In: Hoskins JD, ed. Veterinary pediatrics: dogs and cats from birth to six months. 2nd ed.

Philadelphia: W. B. Saunders, 1995: 133–87.

5. Mahler S, Williams G. Preservation of the fistula for reconstruction of the anal canal and the anus in atresia ani and rectovestibular fistula in 2 dogs. Vet Surg 2005; 34: 148–52.

6. Hare WSD. Anus vestibularis in a young bitch. Can J Comp Med 1959; 23 (9): 278–81.

7. Wilson CF, Clifford DH. Perineoplasty for anovaginal cleft in a dog. J Am Vet Med Assoc 1971; 159: 871–5.

8. Seim HB III. Diseases of the anus and rectum. In: Kirk RW, ed. Current veterinary therapy IX. Philadelphia: W. B. Saunders, 1986: 916–21.

9. Marretta SM, Matthiesen DT. Problems associated with the surgical treatment of diseases involving the perineal region. Probl Vet Med 1989; 1: 215–42.

10. Kochankov D, Vassilev N, Parvanov P. Diseases of the genitals in dogs and cats. Stara Zagora: Aria, 1998: 150.

11. Prassinos NN, Papazoglou LG, Adamama - Moraitou KK, et al. Congenital anorectal abnormalities in six dogs. Vet Rec 2003; 153: 81–5.

12. Romagnoli S, Schlafer DH. Disorders of sexual differentiation in puppies and kittens: a diagnostic and clinical approach. Vet Clin North Am Small Anim Pract 2006; 36: 573–606.

13. Mostachio GQ, Vicente WRR, Cardilli DJ, et al. Anovulvar cleft and vaginal prolapse-hyperplasia in a bitch. J Small Anim Pract 2007; 48: 713–5.

14. Lorenz M, Mark Neer T, DeMars P. Small animal medical diagnosis. 3rd ed. Ames: Wiley-Blackwell, 2009: 329.

15. Holt P. Anal and perianal surgery in dogs and cats. In Pract 1985; 7 (3): 82–9.

16. Hosgood G, Hoskins JD. Small animal pediatric medicine and surgery. Oxford: Butterworth Heinemann , 1998: 103.

17. Wykes PM, Olson PN. Vagina, vestibule, and vulva. In: Slatter D, ed. Textbook of small animal surgery. 3rd ed. Vol. 2. Philadelphia: W. B. Saunders, 2003: 1502–10.

18. Aronson L. Rectum and anus. In: Slatter D, ed. Textbook of small animal surgery. 3rd ed. Vol. 2. Philadelphia: W. B. Saunders, 2003: 682–6.

19. White RN. Surgery of the genital tract. In: Simpson G, England G, Harvey M, eds. Manual of small animal reproduction and neonatology. Gloucester: British Small Animal Veterinary Association, 1998: 179–80.

20. Burt TJ, Smith CW. Vulvovaginal cleft in a dog. J Am Anim Hosp Assoc 1975; 11: 774–7.

ANOGENITALNA RAZPOKA PRI PSICI – KLINIČNI PRIMER

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Povzetek: Na kliniko za male živali na Veterinarski fakulteti Univerze Trakia v Stari Zagori je bila napotena 4-mesečna psička pasme pug z znaki težkega in bolečega uriniranja, pordelosti in otekanja zunanjih spolovil, ki so se pojavljali od enega meseca starosti naprej. Lastnik je opazil težave nekaj dni po rojstvu mladiča, vendar ni poiskal veterinarske pomoči, ker pri psički ni opazil nobenih znakov nelagodja. Fizični pregled ni pokazal nobenih odstopanj od normalnega stanja. S pregledom zunanjih spolovil smo ugotovili nepopolno zaporo dorzalnega stika med nožnico in zadnjikom, izpostavljeno, oteklo in pordelo nožnično ustje in klitoris. Postavili smo diagnozo prirojenega nepopolnega zaprtja anogenitalnega prostora in nepravilnost kirurško popravili. Dorzalno steno nožničnega ustja smo naredili s perineoplastično operacijo v obliki črke V. Na koži med zadnjikom in dorzalnim robom nožnice smo naredili zarezo v obliki narobe obrnjene črke V, nato smo odstranili tkivo med robom kože in sluznico ustja. Steno nožničnega ustja smo zašili s prekinjenim šivom z absorptivnim materialom iz 2-0 poliglikolične kisline, kožo pa s prekinjenim šivom z neabsorptivnim materialom. Operacija je popolnoma uspela, psička ni imela po operaciji nikakršnih komplikacij. Po 5 mesecih smo na kontrolnem pregledu ugotovili normalen položaj zunanjih spolovil in anogenitalnega prostora.

Ključne besede: psica; anogenitalna razpoka; perineoplastika

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Case Report

CONCURRENT SCRAPIE AND CHRONIC COENUROSIS IN TWO CHIOS SHEEP

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Summary: Chronic coenurosis and Scrapie are two of the most common diseases of the central nervous system in the small ruminants of Greece and other countries, and both can cause severe financial losses in affected flocks. Clinicopathological examination of animals *in vivo* is not pathognomonic and so these conditions are usually confirmed by necropsy. This study reports, for the first time in the veterinary literature, two cases of the simultaneous presence of Scrapie and chronic coenurosis in two sheep.

Two Chios ewes aged 1.5 and 2 years, respectively, presenting chronic neurological symptoms, were presented to the Farm Animal Clinic of Aristotle University in Thessaloniki. Sheep 2 also had pruritus on the hindlegs. The haematological and biochemical profiles of the two animals were within the normal limits cited in the literature. Both animals were euthanised and necropsied, and were found to have two and one *Coenurus cerebralis* cyst in their brains respectively. As the two animals belonged to flocks reared in Scrapie-affected areas, the two brains were examined both histopathologically and immunohistochemically and were found to be positive for Scrapie. It can therefore be concluded that Scrapie could be present in sheep with coenurosis, especially in those from areas with a high rate of occurrence of Scrapie.

Key words: sheep; Scrapie; chronic coenurosis; diagnosis

Introduction

Chronic coenurosis and Scrapie are two of the most important chronic ovine encephalopathies, causing losses in sheep flocks in Greece and other countries, with severe financial consequences. In addition, Scrapie belongs to the disease-group of transmissible spongiform encephalopathies (TSEs) and thus has a possible zoonotic impact; any diagnosis is thus required to be reported to the appropriate public health authorities of the European Union (1, 2, 3, 4).

Coenurosis is a parasitic disease caused by *Coenurus cerebralis*, the larval stage of the taenia *Multiceps multiceps* (*Taenia multiceps*) that inhabits the small intestine of dogs and wild canids, the definitive hosts. Two clinical forms of the disease have been described in sheep; the chronic form, which is the most common and is usually observed in animals aged between 6 and 18 months, although it has been described in older animals (1, 4) and the acute form, which is more rarely observed and usually affects animals younger than 1 year (5). Chronic coenurosis presents with an insidious onset and slow progressive focal lesion of the brain (4).

Scrapie is a chronic, progressive and invariably fatal neurodegenerative disorder naturally affecting sheep and goats. It is caused by a prion and is usually observed in animals older than 2 years old, but it has also been diagnosed in younger animals.

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The first signs are usually behavioural changes that are most readily evident to shepherds. These early signs progress to a more definite neurological illness frequently characterized by signs of pruritus and ataxia, one of which usually dominates the clinical course (3, 6).

Clinically, these two encephalopathies are not easily distinguishable other than their different age predilection and the fact that Scrapie is often accompanied by pruritus. As such, diagnosis is based upon necropsy findings or the successful response to surgical treatment in chronic coenurosis or brain histopathological examination in cases of Scrapie. Neither of these diseases can be diagnosed ante-mortem with routine paraclinical examinations *in vivo* (3, 4, 7).

To the best of our knowledge, there has been no report to date describing the concurrence of coenurosis and Scrapie infections in sheep exhibiting neurological symptoms. This study describes the first known cases of coenurosis and Scrapie occurring simultaneously in two sheep belonging to separate flocks.

Materials and Methods

Case history

Two sheep of the Chios breed were presented with chronic neurological symptoms to the Farm Animal Clinic of the Faculty of Veterinary Medicine at the Aristotle University, Thessaloniki, Greece. They came from two different flocks in Northern Greece, neither of which had a history of coenurosis or Scrapie, although both were located in areas of high Scrapie prevalence.

Clinical examination

A thorough clinical and neurological examination of both animals was conducted (8, 9).

Haematological examination

Haematological examinations were conducted using an automated hematology analyser (ADVIA 120, Siemens).

Blood biochemistry

Blood serum samples were examined for glucose, blood urea nitrogen (BUN), creatinine, creatine kinase (CK), total and direct bilirubin, as well as calcium and phosphate, using a clinical chemistry analyser (Flexor E, Vital Scientific).

Maedi-Visna antibodies

Serum samples were examined for the presence of SRLV specific antibodies using the CHEKIT-CAEV/MVV ELISA test kit (IDEXX, Switzerland).

Post-mortem examination

The two animals were euthanased and a full necropsy was conducted.

Histopathology and immunohistochemistry

Tissue samples were taken from the brains of both sheep for histopathological evaluation. The samples were fixed in 10% formol saline and, using standard methods, the tissues were blocked in paraffin and cut into 4–5-μm thick sections. These tissue sections were stained with haematoxylin-eosin (HE), Kluver-barrera stain and periodic acid Schiff stain (PAS) for histopathological examination. Moreover, in sections from the brainstem, at the level of the obex, immunohistochemical staining for PrP^{Sc} detection was performed using the labeled streptavidin avidin-biotin peroxidase method (LSAB kit, DAKO) and applying the monoclonal antibody anti-PrP 2G11. An antigen unmasking pretreatment was carried out in a bath of 87% formic acid for 10 min and hydrated autoclaving at 121 °C for 20 min at 2 atm (12). The staining pattern was revealed by applying 3-3' diaminobenzidine (DAB) as a chromogen.

Parasitological examination

The cysts were examined and identified according to the protocol used in previous studies (13, 14).

Results

Clinical findings

Sheep 1 was an 18-month-old female displaying weight loss, although it had a normal appetite and was afebrile, and its mucosal membranes were normal. It presented with ataxia and epileptiform seizures for 35 days that had gradually worsened. Sheep 2 was a 2-year-old female with a normal temperature, mucosal membranes and appetite. It presented with ataxia, blindness and pruritus on the hindlegs for 30 days that had gradually worsened.

Haematological findings

Haematological findings were within the normal limits cited in the literature for Chios sheep (10).

Biochemical findings

Serum biochemical findings were within the normal limits cited in the literature for Chios sheep (11).

Maedi-Visna examination

Sheep 2 was found to be seropositive for Maedi-Visna infection, while sheep 1 was seronegative.

Gross lesions

On postmortem examination of sheep 1 a cyst containing clear fluid and numerous scolices was detected. The cyst covered part of the brainstem, the cerebellum and the left cerebral hemisphere. Coronal sectioning of the brain revealed that part of the cyst was detected within the dilated left lateral ventricle. A 3 cm-diameter, fluid-filled superficial cyst with white clusters of scolices was also found. A parasitic cyst was also found in the left cerebral hemisphere of sheep 2.

Histology and imminohistochemistry

Histologically, the brain tissue of the affected hemisphere adjacent to the cyst revealed typical pressure atrophy lesions of the cerebral grey and white matter such as degeneration and atrophy of neurons, neuronophagia, satellitosis,

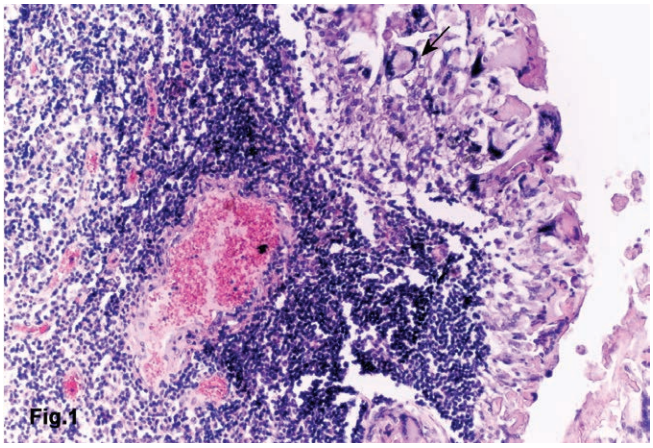

Figure 1: Cerebellum. Periparasitic area characterized by granulomatous inflammatory reaction and necrosis. Presence of foreign body giant cell (arrow). HE x 200

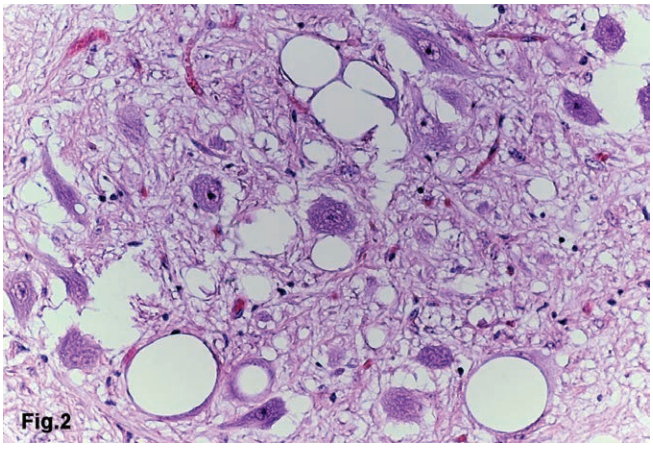

Figure 2: Obex, dorsal motor nucleus of the vagus nerve. Typical pattern of neuropil and neuronal vacuolation. HE x320

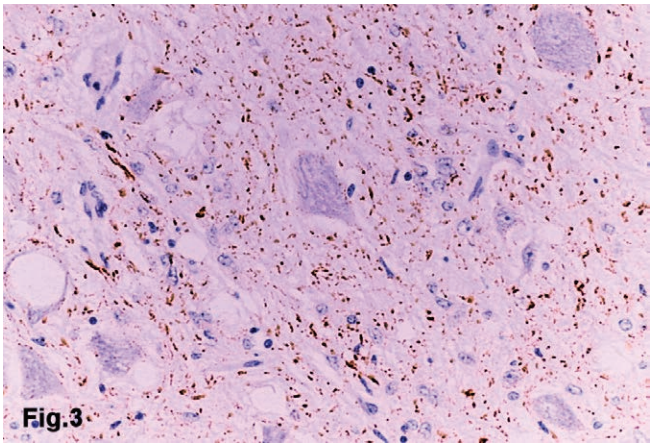

Figure 3: Obex, dorsal motor nucleus of the vagus nerve. Intraneuronal immunolabelling of PrP^{sc} as well as fine punctuate and linear deposits in the neuropil. IHC, LASB, x 320

demyelination, perivascular cuffing or infiltration of mononuclear inflammatory cells, non purulent meningitis, diffuse microgliosis and astrocytosis. The cyst was surrounded by granulomatous inflammation (Figure 1). Blood vessels of medulla oblongata, mesencephalon and choroid plexus in the fourth ventricle were infiltrated by lymphocytes. Neuropil vacuolation and neuronal perikaryonal vacuolation were also found in the dorsal motor nucleus of the vagus nerve at the level of the obex (Figure 2). Immunolabeling of the brainstem, at the level of the obex revealed the following patterns of PrP^{Sc} accumulation: intaneuronal, fine punctuate and linear in the neuropil of the dorsal motor nucleus of the vagus nerve (Figure 3).

Parasitological findings

The cysts that were found in the two sheep brains were identified as *Coenurus cerebralis*.

Discussion

In this study, typical clinical, macroscopic and microscopic findings of both cerebral coenurosis and Scrapie were found occurring simultaneously in the same animal. The differential diagnosis included the three main chronic sheep encephalopathies: chronic coenurosis, Scrapie and Visna, as well as other rarely observed conditions, such as brain abscess or neoplasms that occupy the space within the cranium and can have similar clinical manifestations (4, 7). Based on the clinicopathological findings of both of the living animals before necropsy, sheep 1 was most likely suffering from chronic coenurosis, as it was young and also non-pruritic (4), while sheep 2 at 2 years was probably affected with Scrapie, as it had pruritus possibly complicated by the presence of Visna, as it was also seropositive for this lentivirus. The diagnosis of coenurosis was confirmed by the necropsy findings; histopathological and immunohistochemical examination of the brain excluded the presence of Visna and confirmed Scrapie infection (4, 7).

It is notable that coenurosis may be treated relatively easily surgically and usually has a good prognosis with a high percentage of affected animals recovering quickly (1). Scrapie, in contrast, is an incurable condition and whole flocks should be destroyed where prevalence is high, as required by the EU regulation (3, 7). As such, the suggested course in cases of the co-existence of these two conditions is euthanasia of the affected animal.

In conclusion, in areas or herds with a high prevalence of Scrapie, it is useful to take into account its possible co-existence with chronic coenurosis, and to consider Scrapie as a possible cause of the failure of chronic coenurosis treament.

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References

1. Komnenou A, Argyroudis S, Giadinis N, Dessiris A. Surgical treatment of coenurosis (gid) in sheep. Vet Rec 2000; 147: 242–4.

2. Leontides S, Psychas V, Argyroudis S, et al. A survey of more than 11 years of neurologic diseases of ruminants with special reference to transmissible spongiform encephalopathies (TSEs) in Greece. J Vet Med B 2000; 47: 303–9.

3. Jeffrey M, González L. Scrapie. In: Aitken ID, ed. Diseases of sheep. 4th ed. Oxford: Blackwell Science, 2007: 241–50.

4. Scott PR. Other nervous diseases. In: Aitken ID, ed. Diseases of sheep. 4th ed. Oxford: Blackwell Science, 2007: 259–62.

5. Giadinis ND, Psychas V, Polizopoulou Z, et al. Acute coenurosis of dairy sheep from 11 flocks in Greece. N Z Vet J 2012; 60: 247–53.

6. González L, Dagleish MP, Bellworthy SJ, et al. Postmortem diagnosis of preclinical and clinical scrapie in sheep by the detection of disease-associated PrP in their rectal mucosa. Vet Rec 2006; 158: 325-331.

7. Sargison N. Sheep flock health: a planned approach. Oxford: Blackwell Publishing, 2008.

8. Pugh DG. Sheep and goat medicine. Philadelphia: WB Saunders, 2002.

9. Giadinis ND, Panousis NK, Polyzopoulou Z, Karatzias H. Neurological examination of small ruminants. J Hell Vet Med Soc 2006; 57: 11–8.

10. Panousis N, Kritsepi-Konstantinou M, Giadinis ND, Kalaitzakis E, Polizopoulou Z, Karatzias H. Haematology values and effect of

age and reproductive stage on haematological parameters of Chios sheep. J Hell Vet Med Soc 2007; 58: 124–36.

11. Roubies N, Panousis N, Fytianou A, Katsoulos PD, Giadinis N, Karatzias H. Effects of age and reproductive stage on certain serum biochemical parameters of Chios sheep under Greek rearing conditions. J Vet Med A 2006; 53: 277–81.

12. Haritani M, Spencer YI, Wells GA. Hydrate autoclave pretreatment enhancement of prion protein immunoreactivity in formalin fixed bovine spongiform encephalopathy-affected brain. Acta Neuropathol 1994; 87: 86–90.

13. Giadinis ND, Brellou G, Pourliotis K, et al. Coenurosis in a beef cattle herd in Greece. Vet Rec 2007; 161: 197–8.

14. Giadinis ND, Papazahariadou M, Polizopoulou Z, Panousis N, Karatzias H. Cerebellar dysfunction in a calf with chronic coenurosis. Vet Rec 2009; 164: 505–6.

ISTOČASNA PRISOTNOST PRASKAVCA IN KRONIČNE CENUROZE PRI DVEH OVCAH PASME CHIOS

N. Papaioannou, N. D. Giadinis, P. Tsaousi

Povzetek: Kronična cenuroza in praskavec sta dve izmed najbolj pogostih bolezni centralnega živčnega sistema pri drobnici v Grčiji in mnogih drugih državah, obe povzročata velike finančne izgube v prizadetih čredah. S kliničnim patološkim pregledom živali je bolezen težko diagnosticirati, diagnosticira se šele z obdukcijo. V tej študiji prvič v veterinarski literaturi poročamo o prisotnosti obeh bolezni pri dveh ovcah.

Na kliniko Farm Animal Clinic na Aristotle University v Solunu sta bili pripeljani dve ovci pasme chios, stari 1,5 in 2 leti, s kroničnimi nevrološkimi simptomi. Pri eni je bilo prisotno tudi srbenje na zadnjih nogah. Hematološki in biokemijski profil sta bila pri obeh živalih v normalnih mejah, navedenih v literaturi. Obe sta bili evtanazirani in z obdukcijo je bila v možganih ugotovljena prisotnost cist *Coenurus cerebralis*. Ker sta bili iz čred, vzrejenih v območju praskavca, smo možgane analizirali s histološkimi in imunohistološkimi preiskavami na prisotnost praskavca in ga v obeh primerih tudi potrdili. Zato je mogoče sklepati, da je pri ovcah s cenurozo, še zlasti v območjih z visoko stopnjo pojavljanja praskavca, možno pričakovati tudi prisotnost praskavca.

Ključne besede: ovce; praskavec; kronična cenuroza; diagnoza

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