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Evaluation of tumor necrosis Factor Alpha, Interferon Gamma, Procalcitonin and Neopterin levels in *Brucella* seropositive cattle

Evaluación de los niveles de factor de necrosis tumoral alfa, interferón gamma, procalcitonina y neopterina en bovinos seropositivos para *Brucella*

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ABSTRACT

Brucellosis is a zoonotic disease that affects a large number of people and animals, causing physical disability, workforce loss and significant economic losses in the livestock industry. In the current study, it was aimed to determine and compare the levels of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), Procalcitonin (PCT) and Neopterin in the blood serums of cattle with brucellosis and vaccinated against brucellosis. The materials of this study consisted of a total 48 blood serums belonging to three basic groups, each consisting of 16 animals. Disease group (1st group) were divided into two subgrups each consisting of 8 animals that 21st day after abortion and seropositive 7 months pregnant, the vaccinated (2nd group) and the control (3rd group) groups were divided into two subgroups, each consisting of 8 animals that gave birth 21 days ago and 7 months pregnant. IFN-γ and PCT levels were determined by sandwich enzyme immunoassay, TNF-α and Neopterin levels were determined using competitive inhibition enzyme immunoassay method by using ELISA device. In this study, TNF-α, PCT and Neopterin levels measured in the blood serums of the *Brucella* seropositive (1st), conjunctival *Brucella* abortus S19 vaccine administered (2nd) and unvaccinated *Brucella* seronegative control groups were compared and no significant difference could be determined between the subgroups of the groups ($P>0.05$). There were a significant differences between 1st, 2nd, and 3rd groups ($P<0.05$). IFN-γ levels determined in the blood serums of 1st, 2nd and 3rd groups were compared and nosignificant differences were found between the subgroups of 2nd and 3rd groups ($P>0.05$), but there were a significant differences between the subgroups of the 1st group ($P<0.05$). Similarly, a significant differences were determined between 1st, 2nd and 3rd groups in terms of IFN-γ levels ($P<0.05$). As a result, it was thought that detecting very high serum TNF-α, IFN-γ, neopterin levels in cattle with brucellosis would be helpful in the diagnosis and follow-up of brucellosis. However, it was concluded that there is a need for controlled studies comparing more herds with brucellosis to determine whether the relevant cytokines can be used in the diagnosis of brucellosis.

Key words: Brucellosis; tumor necrosis factor alpha; interferon gamma; procalcitonin; neopterin

RESUMEN

La brucelosis es una enfermedad zoonótica que afecta a un gran número de personas y animales, provocando discapacidad física, pérdida de mano de obra e importantes pérdidas económicas en la industria ganadera. En este estudio se tuvo como objetivo determinar y comparar los niveles de factor de necrosis tumoral alfa (TNF-α), interferón gamma (IFN-γ), procalcitonina (PCT) y neopterina en el suero sanguíneo de bovinos, tanto con brucelosis como vacunados contra la brucelosis. Los materiales de este estudio consistieron en un total de 48 sueros sanguíneos pertenecientes a tres grupos básicos, cada uno compuesto por 16 animales. El grupo de enfermedad (1er grupo) se dividió en dos subgrupos, cada uno compuesto por 8 animales, uno 21 días después del aborto y el otro, seropositivos a los 7 meses de gestación. Los grupos vacunado (2do grupo) y control (3er grupo) se dividieron en dos subgrupos, cada uno compuesto por 8 animales, uno en el que parieron 21 días antes y en el otro con 7 meses de gestación. Mediante el uso de un dispositivo ELISA, los niveles de IFN-γ y PCT se determinaron con un inmunoensayo enzimático tipo sándwich, mientras que los niveles de TNF-α y neopterina se determinaron a través del método de inmunoensayo enzimático de inhibición competitiva. En este estudio, se compararon los niveles de TNF-α, PCT y neopterina medidos en el suero sanguíneo de los grupos de control seropositivos a *Brucella* (1ro), a los que se les administró la vacuna conjuctival *Brucella abortus* S19 de la *Brucella* (2do) y seronegativos en *Brucella* no vacunados, no pudiéndose determinar una diferencia significativa entre los subgrupos ($P>0,05$). Hubo diferencias significativas entre los grupos 1, 2 y 3 ($P<0,05$). Se compararon los niveles de IFN-γ en el suero sanguíneo de los grupos 1, 2 y 3, y no se encontraron diferencias significativas entre los subgrupos del 2do y 3er grupo ($P>0,05$) pero sí hubo diferencias significativas entre los subgrupos del 1er grupo ($P<0,05$). Del mismo modo, en referencia a los niveles de IFN-γ, se determinaron diferencias significativas entre los grupos 1, 2 y 3 ($P<0,05$). Como resultado, se pensó que la detección de niveles séricos muy altos de TNF-α, IFN-γ y neopterina en ganado bovino con brucelosis sería útil en el diagnóstico y seguimiento de la brucelosis. Sin embargo, se concluyó que existe la necesidad de estudios controlados que comparen más rebaños con brucelosis para determinar si las citoquinas relevantes pueden usarse en el diagnóstico de brucelosis.

Palabras clave: Brucelosis; factor de necrosis tumoral alfa; interferón gamma; procalcitonina; neopterina

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INTRODUCTION

Although brucellosis is basically an animal disease, it is also known as one of the most important zoonotic diseases, since more than 500,000 human cases are reported Worldwide every year [1]. Brucellosis affects a large number of people, causing physical disability and workforce loss as well as causing significant economic losses in the livestock industry and negatively affecting the sustainable livestock production [2, 3].

Brucella species are Gram–negative, facultative intracellular bacteria, in the form of cocci, coccobacillus or short rods, 0.5–0.7 µm in width and 0.6–1.5 µm in length. The edges are slightly convex and the ends are rounded. They are appear singly or in pairs, short chains or small clusters on stained preparations. Sometimes the agents can be seen in the form of 3–5 chains in preparations made from liquid media [2, 3].

Brucella abortus is the primary agent of infection in the cattle (*Bos taurus and Bos indicus*),and it can also infect buffalo (*Bubalis bubalus*), bison (*Bison bison*), sheep (*Ovis aries*), pig (*Sus scrofa domesticus*), camel (*Camelus*), deer (*Dama*), horse (*Equus caballus*), dog (*Canis lupus familiaris*) and humans [3, 4]. *B. melitensis* may cause bovine brucellosis, and *B. suis* may rarely cause chronic infection of the mammary glands in cattle in some Countries, especially in Southern Europe and Western Asia, where cattle are kept together with sheep and goats [3, 5].

The most significant feature of *Brucella* infections is that the agent can proliferate both in the phagocytic cells of the reticuloendothelial system and non–phagocytic cells such as trophoblasts [6]. Cellular immune response is more important than humoral immune response in brucellosis [7]. T cell subgroups, macrophages, cytokines released from these cells and interaction between cytokines play asignificant role in protective immunity against intracellular pathogens [7, 8].

Neopterin is a cytokine synthesized from monocytes and macrophages as a result of stimulation of interferon–gamma (IFN–γ) released from active T lymphocytes. Neopterin is a sensitive indicator of cellular immunity [9]. IFN–γ is the most significant macrophage stimulating cytokine and are synthesized by natural killer (NK), T helper (Th) and cytotoxic T (Ts) cells. It enhances phagocytosis of macrophages, stimulates Th1 differentiation and prevents Th2 proliferation [10, 11]. Procalcitonin (PCT) is the precursor of calcitonin hormone produced in thyroid C cells and responsible in calcium homeostasis [12]. It has been reported by various investigators that the normal serum levels of PCT increase one hundredfold in human septicemias [12, 13].

T helper (Th1/Th2) stability plays a significant role in the formation of resistance or susceptibility to brucellosis, and cytokines play asignificant role in the pathogenesis of brucellosis [11, 14]. Studies in mice (*Mus musculus*) have shown that type 1 (Th1) cellular immune response is stimulated in brucellosis. Type 1 cellular response progresses under the control of tumor necrosis factor alpha (TNF–α), interferon gamma (IFN–γ), interleukin–12 (IL–12) produced at the beginning of the disease [10, 15].

There are various studies in the field of Human Medicine on the role of cytokines at post–treatment response and follow–up complications in the pathogenesis of brucellosis [7, 10, 16, 17, 18, 19]. However, studies on farm animals considered as the source of brucellosis are limited [20, 21]. In this study, it was aimed to determine and compare the levels of TNF–α, IFN–γ, PCT and Neopterin in the blood serums of cattle with brucellosis and vaccinated against brucellosis.

MATERIAL AND METHODS

The samples of the study consisted of 48 blood serums belonging to three basic groups each consisting of 16 animals. These groups were the disease group (Brucellosis was diagnosed by the Ministry of Agriculture and Forestry), the vaccinated group (*Brucella abortus* S19 conjunctival vaccination was applied to 4–10 months old animals at a dose of 50 µL), and the control group (blood was taken three times with 2 months intervals and *Brucella* antibody was found negative). The groups divided into two subgroups consisting of 8 animals were as follows; disease group (DG) being 21st day after abortion (DG1) and seropositive 7 months pregnant (DG2), the vaccinated group (VG) and the control group (CG) to gave birth 21 days ago (VG1/CG1) and being 7 months pregnant (VG2/CG2). The blood samples of the study groups were taken from 5 different dairy farms that were diagnosed with brucellosis by the Ministry of Agriculture and Forestry after abortion complaints and from 2 different dairy farms that were certified free from *Brucella* disease between 2014 and 2016. The blood serum samples were stored at -20°C in a deep freezer (Nuve, DF 590, Ankara, Turkey) to be used in serological tests.The presence of *Brucella* antibodies in the CG, DG and VG were determined by Rose Bengal plate agglutination test (RBT) and antibody titers were determined by serum microagglutination test (MAT) according to kit procedures with commercial kits (Rose Bendoll, SAT–A–DOLL) [3]. TNF–α and Neopterin levels were determined by competitive inhibition enzyme immunoassay method, IFN–γ and PCT levels were determined by sandwich enzyme immunoassay according to kit procedures with commercial kits (Cusabio, PRC, USA) using ELISA device (Thermo Fisher, Multiskan FC, USA).

Statistical analysis

Statistical program SPSS (Inc., Chicago, USA 14.0) was used in the analysis of the obtained data. Comparisons between groups were evaluated with ANOVA and post hoc Duncan test. The limit of significance was accepted as $P<0.05$.

RESULTS AND DISCUSSION

In the study, slide agglutination tests were applied to the control group animals 3 times in 2–month periods and all of them were found to be seronegative. The presence of antibodies in the blood serums taken from the DG1 and DG2 were scored with the slide agglutination test, and the antibody titers were determined by the serum microagglutination test. According to the determined values, it was determined that DG1 and DG2 groups were statistically similar among themselves, and VG1 and VG2 groups were statistically similar among themselves ($P>0.05$). However, when the antibody titers were compared between the DG and VG, a statistically important difference was determined ($P<0.05$), values were given in TABLE I.

TNF–α, IFN–γ, PCT and Neopterin levels determined in the study groups were given in TABLE II. In the study, TNF–α, PCT and Neopterin levels determined in the blood serums of cattle belonging to DG, VG and CG were compared. While no statistically important difference could be determined between the subgroups of the groups ($P>0.05$), there was a statistically important difference between the groups ($P<0.05$).

In the study, when the IFN–γ values determined in the blood serums of cattle belonging to DG, VG and CG were compared; While no significant difference could be determined amongthe subgroups of the VG and CG ($P>0.05$), there were a statistically important

TABLE I
Scores determined by slide agglutination test and antibody titers determined by serum microagglutination test of study groups

DG				VG				CG			
DG1		DG2		VG1		VG2		CG1		CG2	
Score	Antibody Titer	Score	Antibody Titer	Score	Antibody Titer	Score	Antibody Titer	Score	Antibody Titer	Score	Antibody Titer
++++	1/320	+++	1/160	+	1/10	+	1/10	–	–	–	–
+++	1/320	+++	1/320	+	1/20	+	1/20	–	–	–	–
+++	1/160	+++	1/160	+	1/20	+	1/10	–	–	–	–
++++	1/320	+++	1/320	++	1/20	+	1/20	–	–	–	–
++++	1/320	+++	1/160	+	1/10	+	1/10	–	–	–	–
+++	1/320	++	1/160	+	1/20	+	1/20	–	–	–	–
+++	1/320	+++	1/320	+	1/20	+	1/10	–	–	–	–
++++	1/160	+++	1/160	+	1/10	+	1/10	–	–	–	–
++++	1/320	+++	1/160	+	1/10	+	1/10	–	–	–	–

(DG; Disease group, DG1; 21st day after abortion, DG2; seropositive 7 months pregnant, VG; vaccinated group, CG; control group, VG1/ CG1; gave birth 21 days ago, VG2/ CG2; 7 months pregnant, N; Negative)

TABLE II
Averages and standard deviations of TNF–α, IFN–γ, PCT and neopterin levels measured in study groups

Biomarkers	DG		VG		CG	
	DG1	DG2	VG1	VG2	CG1	CG2
TNF–α (ng·mL^{-1})	4.51 ± 1.58^{a}	4.56 ± 1.64^{a}	1.94 ± 0.38^{b}	1.83 ± 0.26^{b}	0.92 ± 0.24^{c}	0.88 ± 0.12^{c}
IFN–γ (ng·mL^{-1})	633.04 ± 246.53^{a}	494.03 ± 197.84^{b}	140.43 ± 60.42^{c}	141.54 ± 54.28^{c}	48.14 ± 12.14^{d}	48.32 ± 12.28^{d}
PCT (ng·mL^{-1})	139.24 ± 45.89^{a}	31.86 ± 4.44^{b}	30.9 ± 4.24^{b}	31.51 ± 4.38^{b}	30.71 ± 3.62^{b}	28.88 ± 3.02^{b}
Neopterin (ng·mL^{-1})	8.80 ± 2.99^{a}	7.31 ± 2.49^{a}	4.02 ± 1.18^{b}	3.89 ± 1.22^{b}	1.92 ± 0.46^{c}	1.67 ± 0.22^{c}

^{a,b,c} The difference among groups having different letters on the same line were statistically significant (P<0,05).(DG; Disease group, DG1; 21st day after abortion, DG2; seropositive 7 months pregnant, VG; vaccinated group, CG; control group, VG1/CG1; gave birth 21 days ago, VG2/CG2; 7 months pregnant)

differences between the subgroups of the DG (P<0.05). Similarly, when the DG, VG and CG were compared, it was determined that there were a statistically important differences (P<0.05).

Serum agglutination test (SAT) is an agglutination test that can detect IgM antibodies very well, but has a lower specificity in detecting IgG antibodies, since the pH of the antigen prepared with a suspension of the agent in phenol saline is close to neutral, such as 7.2, Although it is a sensitive test, it is recommended to be used in combination with other tests [22]. In this study, as suggested in the literature and stated in the regulation prepared for free farms by the Ministry of Agriculture and Forestry, seronegativity was confirmed by applying *Brucella* microagglutination and slide agglutination tests to control animals 3 times in 2–month periods to determine seronegativity.

The presence of *Brucella* antibodies in the serums of the DG and VG were determined by the slide agglutination test and scored, and the antibody titers were determined by the serum microagglutination test. When the antibody titers were compared between DG and VG, it was noted that there was a statistically important difference (P<0.05). The definitive diagnosis of brucellosis is made by bacterial growth from clinical samples. However, since it is not always possible to bacterial growth, serological tests gain importance in diagnosis.

The TNF–α receptor complex induces many biological activities in the target cell. TNF–α is released from activated T cells and macrophages as a proinflammatory cytokine [23]. Palmer *et al.* [24] vaccinated 10 pregnant cattle with intravenous *B. abortus* RB51, 5 pregnant cattle subcutaneously with *B. abortus* RB51, 5 pregnant cattle subcutaneously with *B. abortus* S19, 2 cattle in the control group injected subcutaneous non–pyrogen solution. They found that placentatitis occurred after 8–12 weeks in intravenously vaccinated cattle, and TNF–α levels increased, and there was no difference among the subcutaneously vaccinated group and the control group. Akbulut *et al.* [19] determined TNF–α levels in 28 brucellosis cases and 20 healthy individuals in their study on humans, and reported that the TNF–α levels were statistically significantly higher in the patient group compared to the control group. Similarly, in the expression study of Sahiwal cattle vaccinated with *Brucella* abortus S19 by Kumar *et al.* [25] was reported that IFN–γ, TNF–α, IL6, and IL10 genes show initial downregulation and then upregulation. In this study, the mean of TNF–α levels measured in blood serums taken from DG1, DG2, VG1, VG2, CG1, CG2 were determined as 4.51, 4.56, 1.94, 1.83, 0.92 and 0.88 ng·mL^{-1},respectively. The levels of TNF–α determined in the CG subgroups are similiar to the levels determined by Ercan *et al.* [26] in healthy cattle. TNF–α averages determined in DG were found to be higher than other groups (VG and CG) and were statistically important (P<0.05).

In this study, the average of IFN-γ levels measured in blood serums taken from seropositive 21 days ago, seropositive 7 months 21 days pregnant vaccinated, 7 months pregnant vaccinated, seronegative who gave birth 21 days ago, and 7 months pregnant seronegative groups were determined as averaged 633.04, 494.03, 140.43, 141.54, 48.14 and 48.32 ng·mL^{-1}, respectively. IFN-γ averages, which were determined as 48.14 ng·mL^{-1} and 48.32 ng·mL^{-1} in the control group, were similiar with the levels determined by Ercan *et al.* [26] in healthy cattle. IFN-γ averages determined in the disease groups were found to be higher compared to the vaccinated groups and control groups, and this difference was statistically important (P<0.05). Similar results obtained in this study with the study of Ahmed *et al.* [7], in which they determined IFN-γ levels in 27 patients with acute brucellosis and 15 healthy adult individuals, IFN-γ levels were found to be statistically significantly higher in the brucellosis group compared to the control group (P<0.05). Diez-Ruiz *et al.* [16] reported that serum IFN-γ and Neopterin levels were found to be significantly higher in patients with brucellosis than in the healthy control group. Akbulut *et al.* [19] compared serum cytokine levels in 35 patients with brucellosis and a control group of 20 people, and reported that the averages of serum IFN-γ and TNF-α levels were higher in patients with brucellosis than in the control group. El-Boshy *et al.* [20] compared *B. abortus* and *B. melitensis* infected camels with healthy camels and reported that they found lower TNF-α and IFN-γ levels in camels with brucellosis. In the study of Odbileg *et al.* [27] in camels, cytokine levels produced by peripheral blood mononuclear cells in response to *B. abortus* S19 vaccine were determined and it was revealed that IFN-γ level increased during the first week after vaccination. They detected low level of TNF-α expression compared to the control group. In this study, TNF-α and IFN-γ levels measured in the serums of the VG were found to be higher than CG. In studies, low TNF-α in brucellosis patients was attributed to the short half-life of TNF-α Ahmed *et al.* [7], while high TNF-α could be explained by its being a proinflammatory mediator and a high IFN-γ level.

It has been shown in different studies that the serum levels of PCT, which is measured below 0.1 ng·mL^{-1} in the blood serums of healthy individuals, increases at least five times in bacterial infections, exceeds 10 ng·mL^{-1} and even exceeds 1,000 ng·mL^{-1} [12, 13, 28]. In this study, the averages of PCT levels in blood serums taken from DG1, DG2, VG1, VG2, CG1, CG2 were determined as 139.24, 31.86, 30.9, 31.51, 30.71 and 28.88 ng·mL^{-1}, respectively. Serum PCT levels determined in the CG, VG and DG2 were similar with the results determined in healthy cattle by Ercan *et al.* [26]. The fact that the PCT level determined in the abortion group was higher than the other groups is consistent with the studies showing that the PCT level increased in bacterial infections [12, 13, 28, 29]. Undetermining difference between the other groups and the control group may be related to the short half-life of PCT.

Neopterin is a cytokine synthesized from monocytes and macrophages as a result of stimulation of IFN-γ released from active T lymphocytes. Neopterin is a sensitive indicator of cellular immunity Ercan *et al.* [26]. Irmak *et al.* [30] investigated the diagnostic value of Neopterin levels in the follow-up of treatment in 20 patients with brucellos is and reported that Neopterin levels could be used in the follow-up of patients with Brucellosis and evaluating the success of treatment. Diez-Ruiz *et al.* [16] reported that serum IFN-γ and Neopterin levels in patients with brucellosis were significantly higher than the healthy control group. Akbulut *et al.* [19] investigated serum neopterin levels in 30 brucellosis and 30 healthy control groups. They reported that serum Neopterin levels in patients with brucellosis were significantly higher than the healthy CG group. In this study, the averages of neopterin levels in blood serums taken from DG1, DG2, VG1, VG2, CG1, CG2 were determined as 8.80, 7.31, 4.02, 3.89, 1.92 and 1.67 ng·mL^{-1}, respectively. The averages of Neopterin, which were determined as 1.92 ng·mL^{-1} and 1.67 ng·mL^{-1} in CG were similar with the results determined in the healthy cattle by Ercan *et al.* [26]. The averages of Neopterin levels in DG were found to be higher than the VG, CG. This difference was statistically important (P<0.05). The determined results are compatible with the literature [16, 19, 30].

There are few studies investigating cytokine levels in farm animals to elucidate the pathogenesis of brucellosis [20, 27]. In this study, serum levels of biological markers such as TNF-α, IFN-γ, Neopterin and PCT, which are used in the diagnosis and prognosis of infectious diseases in human medicine, were tried to be revealed in Brucellosis and *Brucella*-vaccinated cows.

CONCLUSION

In conclusion, although the fact that serum TNF-α, IFN-γ, Neopterin levels were determined to be quite high in cattle with brucellosis is thought to be helpful in the diagnosis and monitoring of brucellosis, it was concluded that there is a need for controlled studies comparing TNF-α, IFN-γ, Neopterin levels in more herds with brucellosis in order to determine whether TNF-α, IFN-γ, Neopterin levels can be used in the diagnosis of brucellosis in the cattle.

Conflict of interest

There is no conflict of interest between the authors.

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