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An Innovative Approach to Treat Brucellosis in Buffaloes

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Abstract

Brucellosis in cattle and buffaloes is caused by *Brucella abortus* and characterized by late term abortions, placentitis, retained placenta, stillbirth, orchitis, and infertility. The experimental study included healthy (A and B groups) and brucellosis positive (C and D groups) buffaloes. The animals in both the groups A and C were given 1) four injections of long acting oxytetracycline (20mg/ kg body weight; BW) intramuscularly (IM) repeated after every 48 hours, 2) seven consecutive injections of streptomycin (13mg/kg BW IM), 3) three consecutive injections of flunixin meglumin (2mg/kg BW IM) for first three days of the treatment followed by three injections (IM) of Selevit for next 3 consecutive days and 4) combined vaccine on 10th day of the treatment. Each animal of group B and D served as negative and positive control, respectively. Each animal of the group C and D were positive while animals of the group A and B negative to ELISA on six months post-treatment and PCR on subsequent parturition. However, calves of group C, A and B were negative while calves of group D were positive to ELISA and PCR. In conclusion, chemo-immunotherapy is an effective way to treat *Brucella* infected buffaloes in local circumstances.

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Introduction

Brucellosis in cattle and buffaloes is caused by *Brucella abortus.* The disease is characterized by late abortions, retained placenta, orchitis, birth of weak calves and rarely arthritis. There is shedding of the organisms in body secretions primarily in vaginal discharges and milk [1]. The disease also affects a wide range of other hosts such as sheep, goats, equines, swine, camels, dogs, human, and marines. In sexually mature animals it causes significant reproductive losses. The disease is one of the most destructive trans-boundary animal disease hampering export of animals or animal by-products [2].

The disease persists as contagious and zoonotic worldwide. It has a major impact on the health of animals and human as well as ample socio-economic effects, particularly in countries in which pastoral earnings depend mostly on livestock breeding and dairy products. Emphasis may be given on bio-security along with rapid, early diagnosis and control practices. Control of the disease is the prime step to prevent its transmission. The disease is spreading widely on the dairy farms where vaccination is not being practiced. Mass immunization is the only option to eliminate the disease from farm animals [3].

In recent years, brucellosis was diagnosed among less than one percent dairy herds in Pakistan. On the infected herds, more than 10% animals were positive for the disease and soil of the infected herds was also found positive for the *Brucella* species [4]. Vaccination at calf hood age and biosecurity practices help in controlling the disease and prevent its transmission to other susceptible hosts [5]. Due to poor economy of the country and a large number of dairy animals positive for brucellosis, it is almost impossible to implement test and slaughter policy in the country as in the developed world, so there is need of mass awareness among farmers about the mass vaccination and preventive measures. Keeping in view the devastating role of the disease in emerging dairy industry and poor socioeconomic status of the farmers, the project was planned to investigate the effect of chemo-immunotherapy on the recovery of *Brucella* infected buffaloes.

Materials and Methods

Healthy (n=10) and aborted (last trimester) (n=10) buffaloes were selected from a dairy farm in the sub-urban area of Lahore, Pakistan and were divided into 4 groups of 5 buffaloes each viz. (A, B=healthy buffaloes; C, D= Brucella positive buffaloes). Blood sample (4 mL) from each buffalo of each group was collected from jugular vein by disposable syringes (5 ml) using 1.5"x 23G needle and were poured to properly labeled gel clot activator vacutainer (yellow toped). The samples were transported to Internationally Accredited University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences (UVAS), Lahore, in the ice box and centrifuged at 10000 rpm for 2 minutes and clear straw-colored supernatant (serum) was collected in cryotubes for storage at -20^{o}C. Serum of each of the five animals of either group A and B were negative while those of groups C and D were positive for brucellosis through Rose Bengal plate test (RBPT) and Enzyme-Linked Immunosorbent Assay (ELISA) [6]. Animals in groups A and C were given 1) four injections of long acting oxy-tetracycline (Fatro) @ 20mg/kg BW intramuscularly (IM) and repeated after every 48 hours (1, 3, 5 and 7 days), 2) streptomycin (Nawan) @ 13mg/kg BW IM, for 7 consecutive days (1-7 days), 3) flunixin meglumin (ICI) @ 2mg/kg BW IM for first 3 consecutive days (1, 2 and 3 days) followed by Selevit (Fatro) injection (10 ml/animal, IM) for next 3 consecutive days (5th, 6th and 7th day) and 4) combined vaccine (oil based chemically inactivated Foot and Mouth Disease (FMD) virus and live attenuated rough strain of *B. abortus* RB51) on 10th day post-treatment. Each animal of group B and D served as negative and positive control, respectively.

Antibody titer against *B. abortus* in animals of each group was measured at day 0 (pre-treatment) and at days 30, 90 and 180 (Post-treatment) using ELISA test[7].

At subsequent parturition, Polymerase Chain Reaction (PCR) was performed on each serum sample of all animals of each group (A, B, C, and D) as well as sera of calves of each of the calved animals of each group for detection of the bacterial nucleic acid. Genomic DNA of *Brucella* was extracted from serum sample by using QIAGEN DNA extraction kit (USA) following the instruction of the manufacturer. DNA quantification was carried out by "Thermo Scientific NanoDrop spectrophotometer ND-2000". The PCR was executed by means of Taq PCR master mix (Fermentas, USA) with 2µl of template DNA and 0.75µL of each primer as per manufacturer's instructions. The primers used for *Brucella abortus* detection were Forward Primer: 5-

TGGCTCGGTTGCCAATATCAA-3' (223bp size), Reverse Primer: 5-CGCGCTTGCCTTTCAGGTCTG-3'(223bp size) [8]. Thermocycler (EscoSwift™ Mini Thermal Cycler) was programmed according to the standard conditions.

The effectiveness of a particular treatment protocol was evaluated on the basis of antibody titer (RBPT and ELISA) and antigen detection thorough PCR. Moreover, birth weight and placenta dropping time in forthcoming parturition of the animals was also recorded.

Data was analyzed using repeated measures ANOVA and level of significance between groups was determined by Duncan Multiple Range Test. A probability value ($P<0.05$) was considered as significantly different. Statistical analysis was performed by SPSS version 20.0.

Results

Antibody titer against *B. abortus* of each animal of group A, B, C, and D were determined through indirect ELISA and mean ELISA antibody titers of each group of animals are shown in **Table 1**.

Table 1. Comparison of post-treatment antibody titer between treated and untreated animals

Groups	OD Value at day		
	30	90	180
A	0.202 ± 0.031^{ab}	0.305 ± 0.276^{c}	0.165 ± 0.040
B	0.187 ± 0.012^{a}	0.173 ± 0.049^{ab}	0.162 ± 0.035
C	0.883 ± 0.054^{b}	0.910 ± 0.113^{bc}	0.676 ± 0.193
D	0.885 ± 0.101^{a}	0.904 ± 0.081^{a}	0.866 ± 0.066

Note: The mean values of the table having similar superscript are not significantly different.

The animals (group A) negative to RBPT and treated with antibiotics and vaccinated with oil-based combined *B. abortus* and FMD Vaccine, showed anti-*B. abortus* ELISA antibody titer below 0.6 titer on 30, 90 and 180 days post-treatment. Similarly, animals (group B) negative to RBPT but untreated and un-vaccinated showed anti-*B. abortus* ELISA antibody titer below 0.222 titers on 30, 90 and 180 days from the day of experiment initiation. The animals (group C) positive to RBPT, treated with antibiotics and vaccinated with oil-based combined *B. abortus* and FMD Vaccine, showed anti-*B. abortus* ELISA antibody titer above 0.483 on 30, 90 and 180 days post-treatment. Similarly, animals (group D) positive to RBPT but untreated and un-vaccinated showed anti *B. abortus* ELISA antibody titer above 0.800 on 30, 90 and 180 days from the day of experiment initiation. Difference between cumulative mean anti *B. abortus* ELISA antibody titer of animals of group A and B were not significant ($p>0.05$). Similarly, the antibody titer between animals of group C and D were not significantly different ($p>0.05$). However, there was a significant difference between anti *B. abortus* ELISA antibody titer of RBPT negative (A+B) and positive (C+D) animals ($p<0.05$).

Serum samples of RBPT positive animals (group C and D) when tested through PCR remained positive to the genome of *B. abortus* during the whole experimental period. However, serum samples of calves from treated *B. abortus* positive animals showed the negative result to PCR (**Figure 1**).

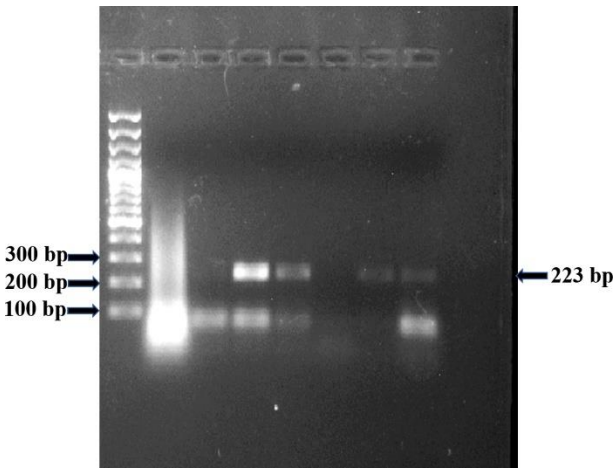

Figure. 1. Gel electrophoresis result of PCR products of *Brucella* bcsp31gene (223bp).
Note: serum sample # 2 and 5 are from calves treated *Brucella* positive buffaloes while serum sample # 3,4 and 6, 7 are from treated and untreated *Brucella* positive animals, respectively.

Birth weight of the calves and dropping time of the placenta on the subsequent parturitions of treated (group A and C) were recorded and shown in **Table 2**.

Table 2. Comparison of birth weight and placental dropping times between brucellosis negative and recovered buffaloes

Parameter (n=4)	**Brucella* negative and treated	***Brucella* positive and treated	P-value
Birth weight (Kg)	32.67 ± 0.882	31.33 ± 1.229	0.260
Placenta dropping time (Hours)	3.25 ± 0.577	3.50 ± 1.302	0.302

Note: Non-treated *Brucella* positive animals did not conceive.
* The calves were from *Brucella* negative (healthy) but treated animals.
**The calves were from *Brucella* positive (infected) but treated animals.

Birth weight between calves of *Brucella* negative but treated animals was not significantly different from the calves of *Brucella* positive but treated animals ($p>0.05$). Dropping time of placenta of *Brucella* negative but treated animals was not significantly different from the dropping time of *Brucella* positive but treated animals ($p>0.05$).

Discussion

Brucella abortus causes abortion in a wide range of hosts including cattle, swine, sheep, goats, camels, dogs and mammals of marine. Establishment of the disease depends upon number of *Brucella* organism, virulence of the organism, susceptibility and age of the host. Non-opsonized organisms enter into macrophages through interaction between bacterial lipopolysacchride (LPS) and membrane of the host cell. The bacteria resist to respiratory burst metabolites of the phagosome and acidic pH, secrete cyclic beta -1, 2-glucans and inserts in the outer side of phagosome membrane thus inhibiting fusion with lysosomes. Lack of phagosome-lysosomal fusion favors the bacterial escape in the cytoplasm for its multiplication [9]. After release from macrophages, the bacteria disseminate within host body through lymphatic system and blood (bacteremia) and localize in susceptible sites such as gravid uterus, supra-mammary lymph nodes, developing fetus (female), epididymis and testicles (male). Presence of erythritol sugar in the uterus supports the bacterial growth outside the cells[10]. Moreover, immuno-compromization of the host by Aflatoxin in ration, endoparasites, ectoparasites, hemo-parasites and many other biological agents potentiate the susceptibility of the host[11]. In *Brucella* positive animals when it was screened against these factors were also found positive for *Theileria spp.* so were treated using Butalex injection. After having recovery from theileriosis, medication of the animals with oxytetracycline (LA) and streptomycin might have inhibited the bacterial growth in intercellular spaces of target organs of the body. As a combination of the antibiotics is effective against the field isolates of *Brucella abortus*, so is commonly used to treat animals suffering from brucellosis [12]. However, such chemotherapy may not inactivate the bacteria hiding in the macrophages. It is pertinent to mention here that chemotherapy over a long period of time is tiring, uneconomical, stressful, and a public health concern due to the antibiotic residues in milk.

On the other hand, because the life span of mammalian macrophages is more than 90 days[13] so after the routine programmed death of the macrophages, the bacteria may be released out and cause bacteremia and next phase of pyrexia. In the present study, infected animals were also injected with non-steroidal anti-inflammatory drugs (NSAID) that eliminate the inflammation, alleviate the pains, and cease production of the inflammatory cytokines that are thought to be a cause of immunosuppression [14]. The NSAID treatment is followed by medication with vitamin E, selenium, vitamin C and lysine which are immunopotentiating drugs[15]. On 10^{th} day post-treatment, the animals were vaccinated using combined oil-based vaccine having binary ethylene imine (BEI) inactivated FMD virus and live attenuated RB-51 (*Brucella abortus*). The RB-51 bacteria trapped in oil can't leave the injection site to cause any ill effects in adult or pregnant animals. The encapsulated live attenuated RB-51 is engulfed and cleared through antigen presenting cells (APC). The bacterial T cell-independent antigen is directly recognized by specific B cells that undergo the process of humoral immunity production while the bacterial T cell-dependent antigen/immunogens are processed and presented along with self MHC-II antigen (Ia) on the surface of the APC. The specific Th cells recognize the immunogen associated with the MHC-II antigen, undergo a process of blast formation, proliferation and differentiation into effecter and memory cells. The effecter cells secrete IL-2, IL-4, IL-5, INF-γ, etc. The cytokines that directly activate B cells, macrophages, Tc cells, Natural killer cells, etc. [16, 17]. Production of the cytokines is antigen-specific but their action is antigen non-specific so residential macrophages of the infected host are activated and the bacteria entering in such activated macrophages are killed [18]. On screening through RBPT and ELISA, the treated and immunized animals showed anti-*Brucella* ELISA antibody titer during the whole experimental period (**Table 1**). Antigens of the pathogenic *B. abortus* inactivated either by antibiotics or cytokine (γ interferon) activated macrophages in the presence of their specific memory B cells may be responsible for perpetuating the ELISA or RBPT antibody titer [19]. The sera from treated and untreated *Brucella* positive animals were positive when screened through PCR for a universal segment or species-specific segment of the bacterial DNA while those of calves of the treated animals were PCR negative (**Figure 1**). The serum of such animals might have a detectable level of specific DNA of the dead bacteria

for some period after treatment but might have cleared latter on. Negative PCR result of the calves from treated *Brucella* positive animals is an indication that the treated hosts have recovered from the infectious agent. This could be a plausible reason for normalization of calf birth weight and placenta dropping time in subsequent parturition (**Table 2**).

It is concluded that chemotherapy with a combination of antibiotics, NSAIDs and immunopotentiating drugs followed by immunonization with oil-based combined vaccine having inactivated FMD virus and live attenuated RB 51 strain *Brucella* abortus vaccine induced recovery in buffaloes suffering from brucellosis. However, isolation and identification of the pathogen from milk, uterine discharge, placenta and biopsy material of supera mammary lymph nodes of the treated animals to confirm their recovery.

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Conflict of Interest

All authors have disclosed no conflicts of interest.

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