



STUDY ON PREVALENCE OF BACTERIAL CAUSES IN CALVES ARTHRITIS

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ABSTRACT

One of the most common causes of calling in calves and economic losses is septic arthritis and joint diseases. Septic arthritis may occur in several routes: direct trauma, extension from particular infection, contamination of the joint or hematogenously. The aim of this study was to determine bacterial causes of septic arthritis and the most effective antibiotic against the isolated organisms. In this study, 40 crossbreed arthritic calves, in both sexes, up to 3 month-old were examined. After general and clinical examination, blood samples were aseptically collected from jugular vein and examined for routine bacterial culture. Synovial fluid samples were also aspirated aseptically from arthritic limb joints and submitted for routine bacterial culture, and polymerase chain reaction (PCR) assay for *Mycoplasma bovis* (*M. bovis*) detection. Antibiotic sensitivity test was performed in cases with positive bacterial culture. Our results showed, WBC, neutrophils counts and fibrinogen in blood samples were increased significantly ($P < 0.05$). *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Corynebacterium bovis* (*C. bovis*) were isolated from 4 (10%), 6 (15%) and 8 (20%) of blood samples of cases with arthritis, respectively. In synovial fluid analysis, viscosity was decreased in all cases. Total protein, WBC and neutrophils counts were increased and monocytes counts was decreased significantly ($P < 0.05$). In 55% of population, synovial fluid culture was positive. It was included *E. coli*, *C. bovis* and *S. aureus*, 2 (5%), 8 (20%) and 12 (30%) respectively. *M. bovis* was detected by PCR from 8 (20%) of synovial fluid of affected animals. In in-vitro antibiotic sensitivity test, gentamycin was the most effective antibiotic against isolated organism during this study.

Keywords: antibiotic, arthritis, bacterial, calves, polymerase chain reaction.

INTRODUCTION

Arthritis is one of diseases that causes significant economic loss in the cattle industry and its symptom is different degrees of lameness, fever, pain and swelling. Usually the synovial fluid become abnormal and contains large number of white blood cells and pathogens [1-4].

Various pathogens are reported as the cause of arthritis in calves and any animals in different areas of the world [2, 4-8].

The septic arthritis of the joint can be created through 4 ways of blood infection, propagation of infection from an infection center in the body (like hoof abscess) and infection caused by individual [1, 9, 10].

Especially in young animals the joint infection can be created following bacteremia or sepsis. It is determined that calves with hypogammaglobulinemia are more sensitive to the bacteremia and septic arthritis. The synovial fluid was first studied in terms of physical characteristics (color, turbidity and viscosity) [1, 9, 10].

Mycoplasma is a causative agent of contagious pleuropneumonia [11, 12], pericarditis [11], mastitis and various reproductive disorders [11, 12], meningitis [11], keratoconjunctivitis [12], arthritis and synovitis [11], pneumonia and arthritis [13] in bovine species. Arthritis caused by *Mycoplasma* primarily reported in the United States and later observed in Australia [11]. Gain access of the organism to the blood stream and localization in synovial surface resulted in an inflammatory reaction in affected joints and adjacent tissues [11, 12]. Mycoplasmal

arthritis is more common in young cattle but adults including dairy cows are also susceptible to the disease. Long transportation and mixing of cattle at different ages may predispose factors to the disease production [11, 12]. Bovine arthritis can be caused by several of *Mycoplasma* spp. such as *M. bovis*, *M. canadense*, *M. alkalenscens* and *M. bovis genitalium* [11, 12]. Review of literature indicates the importance of *Mycoplasma* spp. as causative agents of bovine arthritis and several diseases. *M. bovis* has been the most common isolate from bovine arthritis [11]. Usually the disease also needs to long-term treatment and paying high medical costs. Therefore sometimes the treatment is not economic due to high costs of the treatment and in result more economic losses are provided [4, 9, 14, 15]. Using inappropriate antibiotic, delay in starting treatment and creation of irreversible lesions in tissues and structure of the joint can be mentioned as causes of the failure in treatment, also [16, 17].

The first diagnosis of this disease is based on clinical symptoms while the differential diagnosis is based on evaluating synovial fluid and culture and using further diagnostic techniques such as, radiology, ultrasonography, fluorescent antibody, gas chromatography [4, 9, 14, 18-20].

So the main purpose of this study is to determine prevalence of bacterial agents in arthritis in calves of the region. Meanwhile in this study the most effective antibiotic in treatment of the mentioned disease is determined by inform the antibiogram test in order to



consider an acceptable route in clinical cases as the first antibiotic selection while the time is saved.

MATERIALS AND METHODS

Study areas and sampling

Samples were collected from referent calves to the Veterinary Clinic of Islamic Azad University of Shahrekord Branch and cattle breeding centers around the Shahrekord, Iran. After general and physical examination, sampling was performed from 40 crossbreed calves younger than three months age that affected to arthritis.

First, joints of limbs were examined in terms of heat, inflammation, swelling, pain and it was tried to obtaining clinical examination confirmation after that the arthritis was proved and the owner satisfaction was achieved. Then blood was collected from jugular vein, in the aseptic condition by needle gauge no.18 and was maintained in laboratory tubes containing anticoagulant substance, EDTA, along with ice and then was issued to the laboratory in order to study hemogram and culture.

Bacterial strains, culture conditions and PCR

The blood sample was cultured on Eosin Methylene Blue (EMB), blood agar, and nutrient agar as liner in the vicinity of the flame and was put in incubator for 24-48 h at the temperature of 37°C. Then the bacterial isolation was performed if the bacterial grow occurred. Also about joints that showed the disease symptoms (heat, inflammation swelling and pain), first hairs of the area was shaved and then the local disinfection was performed. The synovial fluid was aspirated by the needle gauge no.18 for cellular studies, determination of total protein and linear cultures on EMB, blood agar and nutrient agar as mention above. The cultures were incubated for 24-48 h at the temperature of 37°C. Then the bacterial isolation and antibiogram test were performed if the bacterial grow was seen and results were defined as resistance, sensitive and moderate. Also a sample of synovial fluid from each studied calf was issued to the central laboratory to detection *Mycoplasma bovis* (*M. bovis*) in PCR method.

DNA extraction

DNA extraction from samples was using according to the method described by the Laird *et al.* [21] and the total DNA was measured at an optical density of 260 nm.

DNA amplification and detection of PCR products

The PCR reaction mixtures were placed in a thermal cycler (Mastercycler gradient, Eppendorf, Germany). As a forward primer, Mb1 (5'-AAGGTACACCAGCTAACCCAG-3'), described by Ghadersohi *et al.* (1997) [22] was used. The Mbr2 reverse primer (5'-AATGAAGCTACTGATCCAAG-3') was used based on the *M. bovis* variable surface lipoprotein (*Vsp*) genomic region sequence published by Lysnyansky *et al.* (1999) [23].

PCR conditions

PCR was carried out in a total volume of 50 µl, containing 1×, 50 mM KCl, and 200 µM of dNTP, 20 picomoles of each primer, 5 µl of DNA and 2.5 U of Taq polymerase. After 5 min initial denaturation at 94 buffers for a final concentration of 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 35 cycles were performed with the following parameters: 20 sec at 94°C, 20 sec at 52°C, and 1 min at 72°C. After cycling, a final extension was applied for 5 min at 72°C.

Electrophoresis

The PCR products were dissolved in a 1% (w/v) agarose gel containing 1× TBE buffer (100 mM Tris-HCl (pH 8), 90 mM boric acid, and 1 mM Na₂EDTA), stained with an ethidium bromide solution (0.5 µg/ml) and a DNA ladder (Fermentas Co., Germany) used to detect the molecular weight of observed bands and visualized under UV light. Also, were images obtained in UVIdoc gel documentation systems (Uvitec, UK).

Antimicrobial resistance test

Antibiotic susceptibility test was performed by Kirby-Bauer disc diffusion method on Mueller Hinton agar based on recommendations of CLSI (formerly the National Committee for Clinical Laboratory Standards) (NCCLS, 2008) [24]. The following antibiotics were used in this study: streptomycin, linco-spectin, oxitetracycline, ampicillin, penicillin, amoxicillin, enrofloxacin, gentamicin which were purchased from Padtan-Teb Company (Tehran, Iran).

Statistical analysis

Data were transferred to a Microsoft Excel spread sheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using the Statistical Package for the Social Sciences (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

This study was performed for 14 months. The maximum and minimum age in studied calves was 6 and 90 days respectively with the average age of 32 days. Studied calves included 18 (45%) male and 22 (55%) female calves that there was no significant difference between two sexes statistically ($P < 0.05$). In current study, in 8 cases, arthritis was with history and symptoms of other diseases that 8 cases (20%) were with pneumonia, 6 cases (15%) with umbilical cord infection and 2 cases (5%) with diarrhea. The body temperature and heart rate have significant increase compared to normal values in the clinical examination of calves affected to the arthritis ($P < 0.05$).

Number of white blood cells (WBC), neutrophil, band cell and amount of blood fibrinogen showed the significant increase than their normal values but blood lymphocytes showed significant decrease than their normal values ($P < 0.05$). No significant difference was seen in number of red blood cells, monocytes and eosinophils in blood compared to their normal values ($P < 0.05$).



The bacterial agents were isolated from 45% of blood cultures totally. Bacterial agents isolated from blood culture of calves affected to arthritis and their frequency has been presented in the Table-1.

In synovial fluid, for detection of *M. bovis* in PCR methods, 8 samples were positive (Figure-1). *M. bovis* was isolated from 4 pneumonic calves. In detection of *M. bovis* in synovial fluid with PCR methods, 8 samples were positive. In some cases *M. bovis* was available combined with other bacterial agents that these included 4 cases (10%) with *Staphylococcus aureus* (*S. aureus*), 2 cases (5%) with *E. coli* and 2 cases was only *M. bovis*. Bacterial agents were isolated from 55% of synovial fluids culture. Their prevalence has been showed in the Table-2.

Table-1. The prevalence of bacterial agents isolated from blood culture of affected calves.

Bacterial agents	Prevalence (n) (%)
<i>C. bovis</i>	8 (20%)
<i>E. coli</i>	6 (15%)
<i>S. aureus</i>	4 (10%)

Table-2. Prevalence of bacterial agents isolated from the synovial fluid culture of affected calves.

Bacterial agents	Prevalence (n) (%)
<i>C. bovis</i>	8 (20%)
<i>E. coli</i>	2 (5%)
<i>S. aureus</i>	12 (30%)

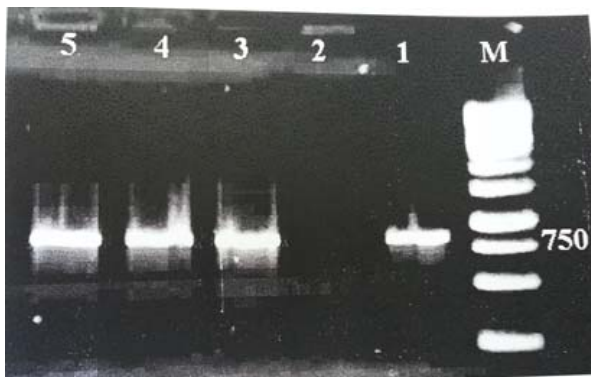


Figure-1. Ethidium bromide-stained 1% agarose gel electrophoresis of PCR products. *M. bovis* were used to amplify a 750-bp. Lane M contained 100-bp DNA marker, lane 2 to 5 contained PCR products (lane 2= negative; lane 3, 4 and 5= positive), and lane 1 contained positive control.

In cases with positive synovial fluid culture, the antibiogram test was performed by using tylosin, streptomycin, linco-spectin, oxitetraciclina, ampicillin, penicillin, amoxicillin, enrofloxacin, gentamicin disc. Results of the biogram test of the synovial fluid were obtained for amoxicillin 62.5% resistant, 25% average and

12.5% sensitive. Results obtained from the antibiogram test of the synovial fluid shown the most suitable effect of the gentamicin on bacterial agents in arthritic calves. In all cases the pathogen shows resistance to the ampicillin and penicillin. The isolated bacteria show moderate reaction against other antibiotic discs in this study.

DISCUSSIONS

Increase in heart rate, body temperature and changes in blood cellularity indicate persistence of infection condition in calves, beside in 8 cases arthritis were with other diseases. In current study, 55% of synovial fluid culture was positive. Frey *et al.* [25], Kent-Lloyd *et al.* [26], Madison *et al.* [27] and McGee [28] reported 40-75% success in isolating infectious agent from synovial fluid culture.

Negative results of synovial fluid culture in cases of infectious arthritis may be provided by presence of some other agents like viral agents or establishment of infectious agents in the synovial membrane, antibiotics consumption, immunity factors and activity of leucocytes, sampling method, condition of transferring the samples to the laboratory and laboratory methods [12, 29].

In the present study bacterial agents isolated from the synovial fluid culture included *S. aureus*, 12 cases (30%), *Corynebacterium bovis* (*C. bovis*), 8 cases (20%) and *E. coli*, 2 case (5%) (Table-2). Prevalence of each of the bacterial agents than other bacterial agents compare to bacterial agents isolated in this study did not show statistical difference. This issue can be resulted by limitedness of studied samples number. Most frequent bacterial agents isolated from arthritic of calves in other reports include: *E.coli*, *Salmonella* spp., *Arcanobacterium*, *Fusobacterium necrophorum*, *Staphylococcus* sp. and *M. bovis* [12, 30-32].

Firth *et al.* [32] and Van plet *et al.* [30, 31] reported that the most frequent agents in new-born arthritis calves had been *Streptococcus* spp. and *Coliforms* and in older calves and cow had been *Actinomyces pyogenes* [30-32]. In this study in 8 cases (20%), *M. bovis* was isolated from synovial fluid by the PCR method.

As observed in Tables 1 and 2, *S. aureus* is isolated from blood in 4 cases and from the joint in 12 cases and *E.coli* in 6 cases from blood and 2 cases from joint. These results can be explained with regard to difference in tissue affinity and pathogenicity between *E. coli* and *S. aureus*. *S. aureus* has high affinity to synovial membrane and can survive intracellular in result it remains for longer time in the joint but *E. coli* is rapidly removed from locality and rarely remains there [12,33]. *S. aureus* is able to distribute in the tissue extensively and create abscess in all organs [34]. In one study after venous injection of *S. aureus* to mice only in 2.5% of population septicemia was observed while majority of mice became affected to septic arthritis. Regarding above results, Bremell *et al.* [35] announced that *S. aureus* remains for a short time in blood but it tends to be located in tissues specially joints. Tissi *et al.* [36] reported, also septic arthritis was observed following venous injection of *Staphylococcus agalactiae* in mice.



Surface proteins of *S. aureus* and their adherence properties can play an important role in pathogenicity of infectious arthritis. These include ability to binding to fibronectin [37, 38] or collagen [39] as well as less specific hydrophobic interactions [40] that causes bacterial adhesion to synovial membrane. Reaction between *Staphylococcus* and collagen and fibronectin are unique and can be effective in maintaining infections process of *Staphylococcal* in bone and joint. Also *S. aureus* can bind dedicatedly to bone sialoprotein [41]. Certainly, ability of *S. aureus* to attach bone is very important in its Pathogenicity and can justify high frequency of joint and bone infection after bacteremia [42].

In some cases *M. bovis* was existed as combined with other infectious agents that this includes 4 cases (10%) of *M. bovis* accompanied by *S. aureus* 2 case (5%) accompanied by *E. coli* and 1 case (5%) was only *M. bovis*. There are some reports based on isolation of only *M. bovis* or combined with other infectious agents in calves' arthritis [43-46]. Dyer *et al.* [47] in USA succeeded to isolate *M. bovis* by the PCR method from synovial fluid in studying two Bisons affected septic arthritis and lameness. One farm in Australia that 30% of cows in the range of 2-3 weeks olds were suffered from lameness and polyarthritis, *Mycoplasma* was reported as the cause of disease [48]. Hammond *et al.*, (2003) [6] succeeded for the first time to isolate *Mycoplasma* from synovial fluid of a giraffe affected to arthritis by the PCR method in Texas. In the present study, 4 of calves who detected *M. bovis* in synovial fluid, were suffered from pneumonia, also *M. bovis* is an agent that can causes pneumonia and lameness in calves. Study of Gagea *et al.* [49] conducted in a farm in Canada, where calves were from arthritis and pneumonia, reported that 46% of calves with pneumonia resulted from *M. bovis* were also suffered to arthritis.

Shiel *et al.* [50] and Alexander *et al.* [51] have presented a report based on isolation of *Mycoplasma* from calves affected to pneumonia and polyarthritis. Anyway *Mycoplasma* infectious arthritis might be without any sign of pneumonia [52].

Adegboye *et al.* [53] believed prevalence of *Mycoplasma* arthritis following pneumonia supports the theory that one of the ways that arthritis resulted from *M. bovis* can be provided is respiratory infection transfer through the air. Although creation of infectious arthritis and pneumonia in calves suckling milk in result of using milk of cows affected to *Mycoplasma* mastitis is also proposed [51].

Byrne *et al.* [54] believe that if other symptoms were not observed in area of interdigital space, hoof or upper limb when performing clinical examination, especially if there is history of livestock purchase or occurrence of lameness after respiratory disease recently must be considered in subtractive diagnosis of lameness.

Results obtained from antibiogram test of synovial fluid in arthritic calves show suitable effect of gentamicin while, in all cases, pathogen showed resistance against penicillin although there is a report based on penetration of penicillin in synovial fluid [12]. Report of Hum *et al.* [48] indicates bacterial resistance in infectious

arthritis against ampicillin, penicillin, streptomycin, erythromycin, sulfonamide, lincomycin and sensitivity of the agent causing infectious arthritis against lincospectin and tetracycline. In the present study resistance level against streptomycin, oxytetracycline and ceftiofur was moderate, indicating inappropriateness of mentioned drugs in treatment of arthritic calves.

Due to presence of different results in this regard, selecting appropriate antibiotic with regard to antibiogram test is a determinant in result of treatment. Anyway in ruminant, antibiotics that are effective on *Coliform*, *Streptococcus* should be used before antibiogram test [55]. Results of this study indicate appropriate effect of gentamicin on agents causing calves' arthritic. Of course it should be noted that results obtained from this study are in laboratory condition and ability to penetration and amount of drug activity in the synovial fluid, synovial fluid pH and type of pathogen are influence the result of antibiotic therapy. Meanwhile infectious agents in fibrin clots will be safe against antibiotic effects [19].

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