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Determination of *Actinobacillus seminis* by Capillary Zone Electrophoresis

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Abstract: The use of sophisticated techniques for the analysis and identification of microorganisms is more and more common; such is the case of capillary zone electrophoresis technique, which allows a quick separation and identification of microorganisms. *Actinobacillus seminis* is a bacteria involved in ovine epididymitis, a disease common to this species and currently, its identification is solely bacteriological and is conducted through a microbiological analysis, which is confusing and slow, since there is not specific biochemical test that distinguishes this bacterium from other bacteria that are also involved in ovine epididymitis, such as *Brucella ovis*, mainly. This work presents pre-treatment results and run conditions of *Actinobacillus seminis* for its identification through capillary zone electrophoresis.

Key words: *A. seminis*, CZE, epididymitis

INTRODUCTION

Contagious ovine epididymitis is defined as infection of the testicle and epididymis in rams. This illness may occur in acute, subacute and chronic phases and injuries are clinically detected due to testicle degeneration and atrophy, resulting in subfertility or sterility of the infected rams (Appuhamy *et al*., 1998). Ovine epididymitis damages young and adult animals causing important losses to the sheep industry. This disease represents a serious reproductive disorder in which marked pathological changes occur in the genital tract of clinically affected rams (Núñez *et al*., 2006). Infectious epididymitis in rams is considered an economically important pathology because of its adverse effects on the male and herd fertility. This pathology has been primarily associated with *Actinobacillus seminis* (*A. seminis*) and *Brucella ovis* (*B. ovis*) infections (Acosta *et al*., 2007).

A. seminis is a pleomorphic coccobacillus, gram-negative, measuring 1 × 1-4 μm, grouped in chains or forming palisades, non-motile and non-sporulated. This pathogen is a facultative anaerobe with fermentative metabolism; its optimum growth occurs when cultures are incubated in an atmosphere of 10% CO_2 at 37°C in media with blood or serum. After 24 h, the bacterium grows in media with blood or serum as small colonies that at 48 h are large and white, of 1-2 mm in diameter (Burgess, 1982; Koneman, 1999). Currently, the diagnostic technique used to identify *A. seminis* is bacteriologic and includes Gram staining and modified Ziehl-Nielsen staining. This technique is performed until the animal shows the characteristic signs of the infection. There is no specific biochemical test to distinguish *A. seminis* from the other bacterial species involved in epididymitis (Núñez *et al*., 2006).

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Detection of microorganisms such as bacteria is very important in bioscience research, medical diagnosis and profiling of some diseases, analysis in food industry and quality control of fermentation processes (Szumski *et al*., 2005). Identification and characterization of microorganisms has historically been based on morphologic, biochemical, serologic, toxigenic and genetic characteristics. Many procedures require the preparation of bacterial cultures, which dramatically lengthens the analysis time. In recent years, much effort has been made to develop high efficiency and fast instrumentation separation techniques for the determination and detection of biological macromolecules and living cells. A variety of techniques exist for the analysis of microorganisms. Each of these methods may determine one or more aspects of a microbial system (identification, quantification or characterization). These techniques include: polymerase chain reaction (PCR) detection, flow cytometry, differential staining, serological methods and recently dielectrophoresis, Field-Flow Fractionation (FFF), combined dielectrophoretic and field-flow fractionation Microsystems and Matrix-Assisted Laser Desorption/ Ionization (MALDI) mass spectrometry. Recently, molecular techniques have come to the forefront as an alternative procedure of microbial detection. They are divided into three types: hybridization, amplification and immunoassay techniques. Hybridization involves the use of fluorescently labelled nucleic acid probes that bind to complementary nucleic acid sequences present in bacteria (Desai and Armstrong, 2003). These complementary nucleic acid strands specifically bind only to the probe. Once the probe binds to the complementary strand of nucleic acid, the fluorescent bacteria can be visualized microscopically. Amplification techniques, such as PCR, extract a small amount of genetic material from the bacteria and amplify it to a level that can be quantified for identification (Desai and Armstrong, 2003). Immunoassay methods make use of the very specific reaction between antigens on the surface of bacteria and the antibodies that the immune system produces in response to them. The time used for these tests is considerably less than that for culture-based methods, in most instances requiring only several hours to complete (Schweickert *et al*., 2004).

Several attempts have been made to adapt chemistry's modern instrumental methods to the characterization and identification of various microbes. This includes various optical spectroscopic techniques and different hyphenated chromatography-mass spectrometry (MS) approaches (Armstrong and Schneiderheinze, 2000). Analytical techniques, in particular Capillary Zone Electrophoresis (CZE), have been used recently to address some of the problems associated with microbial detection and identification methods (Rodríguez and Armstrong, 2004). CZE is an attractive technique because of its fast analysis times and very small sample requirements (which often is the case with microbial samples) (Rodríguez *et al*., 2006).

The optimum conditions that allow quick, opportune and precise identification of *A. seminis* by capillary zone electrophoresis are shown here. This will allow the quick monitoring of sheep infected with this microorganism, reducing the incidence and complications of this infection, which will be reflected on the animal production.

MATERIALS AND METHODS

Materials: Tris (hydroxymethyl) aminomethane (TRIS), boric acid, were acquired from Aldrich (Milwaukee, WI), the bacterial species used was *Actinobacillus seminis* 15768, obtained from the American Type Culture Collection (ATCC) (Manassas, VA), *Brucella* Agar (BA) was obtained from Becton Dickinson (Rutherford, New Jersey).

Bacterial growth: *A. seminis* 15768 was grown in *Brucella agar* for 24 h, suspended in physiological saline solution and centrifuged twice at 6,500 rpm for 5 min. The obtained pellet was resuspended in TBE and submitted to sonication for one minute in an Ultrasonic LC30H (Germany) for complete disintegrate.

Sample preparation: Starting with a 24 h growth, a suspension of *A. seminis* cells was prepared at a concentration of 1.5×10^7 Colony Forming Units (CFU), from which 1 mL was taken to conduct three washes with saline solution 0.85%, in each wash the cells were centrifuged at 6,500 rpm for 5 min. Additionally, 1 mL 1.5×10^7 was centrifuged at 500 rpm; subsequently the supernatant was passed through a sterile nitrocellulose filter (Gibco Laboratories, Carlsbad, California, USA) with a 0.22 μm pore size. Finally, the bacteria were resuspended in 2 mL TBE buffer for 30 min for later analysis. With the objective of determining the detection limit of *A. seminis*, decimal delusions were prepared, to be analyzed by capillary electrophoresis. Cell concentration was determined by colony count in *Brucella* agar plates; 0.5 mL of the washed and filtered bacteria suspension were taken, to each suspension, 40 μL of Mesityl oxide (Sigma Chemical Company, St. Louis, Missouri, USA) 0.01% were added, as a marker of electroosmotic flow, the mixtures were sonified for 1 min. The obtained suspensions were analyzed by CZE.

Zone capillary electrophoresis: The buffer (TBE buffer) was prepared using deionized water with a concentration of 9.0 mM of TRIS, 9.0 mM of boric acid and 0.1 mM of

EDTA. With the aim of determining the optimum buffer pH with which *A. seminis* cells exhibit adequate migration, experiments were conducted varying pH values pH (7.0, 8.0 and 9.0).

The runs were conducted with a Beckman Coulter P/ACE Capillary Electrophoresis (Beckman Coulter, Palo Alto, CA, USA) equipped with a diode array detector. Fused silica capillaries with a total length of 50 and 40 cm to detector, 75 μm of inner diameter (Beckman Coulter, Palo Alto, CA) were used. The analysis conditions were: hydrodynamic injection at 1.0 psi for 10 sec; for the separation, a 20 kV voltage was applied. Prior to the first run of the day, the capillary was activated with NaOH 0.1 M for 10 min at 20 psi and, between runs, washes were made with deionized water (Milli-Q plus, Millipore, France) for 5 min at 20 psi; before the sample's injection, a 5 min conditioning was done with the run buffer. All the experiments were performed at a constant temperature of 25°C and a wave length of 200 nm.

RESULTS

Electrofherograma from *A. seminis* with pretreatment: Figure 1 shows the electropherogram obtained from cells with pretreatment, where a peak corresponding to the electroosmotic flow marker, exhibiting a migration time of 2 min, is identified; on the other hand, *A. seminis* exhibits a migration time of 2.85 min, showing a well-defined and broad peak. According to the exhibited results, we can say that the pretreatment applied eliminates some problems related to the sample's nature, making possible the identification of *A. seminis* through CZE.

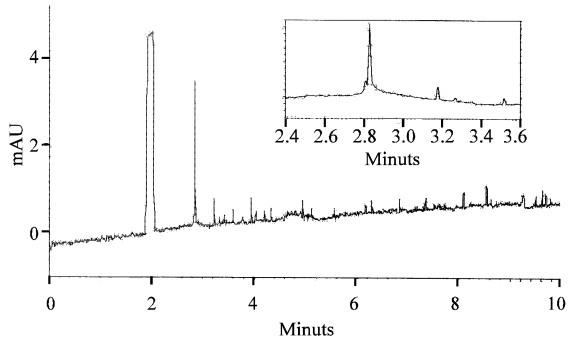

Fig. 1: *A. seminis* bacteria run at a pH of 8.0 with successive washes, the box is an amplification of the peak corresponding to the bacteria (second peak), the first peak is the electroosmotic flow marker. The run conditions were: fused silica capillary with a total length of 50 cm and a effective length of 40 cm, the run buffer was Tris-Borate-EDTA (TBE) pH 8.0. The detection wavelength was 200 nm, applying a 20 kV voltage

Electropherogram obtained with filtering: Figure 2 shows the electropherogram obtained when filtering 1 mL of *A. seminis* bacteria suspension, of 1.5×10^6 CFU, through a nitrocellulose membrane of 0.22 μm; the results indicate an increase in baseline quality, in addition to a much higher and sharper signal.

Detection limit of *A. seminis*: The detection limit of the method proposed in this work for the identification of *A. seminis* is 1.5×10^6 CFU (Fig. 3).

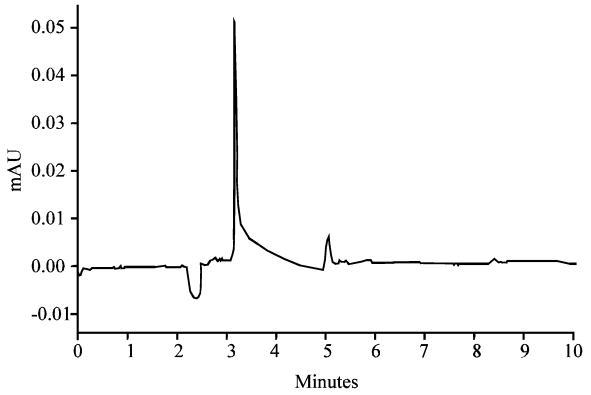

Fig. 2: The electropherogram shows the bacterial run when successive washes are conducted and in addition it is filtered through a 0.22 μm sterile membrane. A well-defined and resolute peak is observed with regards to the electroosmotic flow marker. Run conditions are the same as in Fig. 1

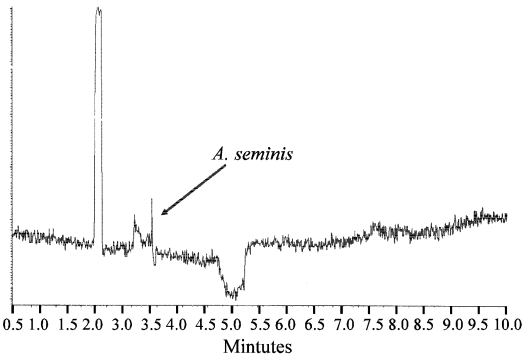

Fig. 3: Determination of the *A. seminis* detection limit with the proposed method, this value is of 1.5×10^6. The value of the signal-noise relation is of 3/1. Run conditions are the same as in Fig. 1

DISCUSSION

In recent years, capillary electrophoresis has become a major alternative for fast, efficient routine separation of molecules with simple preparation. The CZE technique was originally developed for the analysis of molecules, its advantages have been extended to the determination of biological particles such as cells and microorganisms (bacteria and virus) (Taylor and Fernandez-Patron, 2000).

Self-aggregation of cells in an aqueous matrix is a common phenomenon that must be understood and controlled in bacteria analysis (Scheneider *et al*., 2000). Many microorganisms release biomolecules (enzymes and proteins) and these secretions may produce unwanted peaks. Variations of microbial cell wall composition can result from isolation and preparation procedures, growth conditions or age. Moreover, some bacteria may adhere to various surfaces or to other microbes (Szumski *et al*., 2005). Cluster formation of bacterial cells can affect electrophoretic mobilities of bacterial cells as the charge-to-size ratio varies. Also, bacteria cells tend to precipitate and aggregate because of the gravity effect on the cells themselves, which may also cause irreproducible electrophoretic mobilities of bacterial cells. Currently, vortexing and sonication are the most commonly employed means for simple bacterial pretreatment. Different bacteria may have different association strength among cells and so pretreatment of bacteria cannot be transferable. There for, it is essential to understand the optimum pretreatment for each kind of bacteria to obtain a single, sharp peak during electrophoresis (Yu and Li, 2005). Since, the bacteriologic methods that are usually employed in *A. seminis* identification do not provide quick and accurate results, studies were performed so that when conducting pre-treatments to CZE runs, their fast and accurate identification would be achieved. Figure 1 shows the electropherogram obtained from cells with pretreatment, where a peak corresponding to the electroosmotic flow marker, exhibiting a migration time of 2 min, is identified; on the other hand, *A. seminis* exhibits a migration time of 2.85 min, showing a well-defined and broad peak. According to the exhibited results, we can say that the pretreatment applied eliminates some problems related to the sample's nature, making possible the identification of *A. seminis* through CZE.

Figure 2 shows the electropherogram obtained when filtering 1 mL of *A. seminis* bacteria suspension, of 1.5×10^6 CFU, through a nitrocellulose membrane of 0.22 μm; the results indicate an increase in baseline quality, in addition to a much higher and sharper signal. These results show that filtration, in addition to making a more specific clean-up, allows us to make a preconcentration prior to injecting the sample to the capillary, because the sample was resuspended in only 500 μL and so a higher signal is shown, this indicates that the sample's filtration together with the pretreatment make possible the identification of the bacterial sample. The relative standard deviation (RSD) of the effective mobility (m_{eff}) of *A. seminis* was interday RSD=0.47 (n=6, with a pH of 8.0) and intraday RSD=0.76 (n=6 with a pH of 8.0). Therefore, the proposed method allows the clear and precise identification of *A. seminis*.

For the study of pH influence on the analysis, the work of (Palenzuela *et al*., 2004) was considered, who in 2004 reported that Gram-positive bacteria exhibit negative electric charge above pH 5.0, the electrophoretic mobility of Gram-positive bacteria was to pH between 4.0 and 7.0 and they said that the mobility of Gram-negative bacteria changed very little with this pH range. Also, both types of bacteria exhibited virtually constant electrophoretic mobility in the pH range of 7.0-10.0 (17); however, so far, there is no report whatsoever on the electrophoretic mobility of *A. seminis* with different pH values. The effective mobility values, as mentioned by Palenzuela *et al*. (2004) do not exhibit a great difference in this range of pH, but for some reason that we haven't been able to clarify, there is an influence of the run pH with regards to the form of the peak corresponding to the bacterial sample and it is clear that the influence of this factor is indeed a determinant in the identification of *A. seminis* and is therefore an important variable that affects the peak's quality (broad and tall). Finally, we found that the detection limit of the method proposed in this work for the identification of *A. seminis* is 1.5×10^6 CFU (Fig. 3). Studies corresponding to the method's validation in the biological sample are being researched.

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