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Original Research

Ability of Chromogenic Agar, MALDI-TOF, API 20E and 20 Strep Strips, and BBL Crystal Enteric and Gram-Positive Identification Kits to Precisely Identify Common Equine Uterine Pathogens

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ABSTRACT

Bacterial endometritis is a well-recognized cause of subfertility in mares. Clinicians rely on accurate identification of bacteria to determine if the isolate is pathogenic to develop a therapeutic plan. To determine the accuracy of bacterial identification systems, 25 isolates each of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Streptococcus equi* subspecies *zooepidemicus* were evaluated by Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF), chromogenic agar, API bacterial identification system and BBL Crystal bacterial identification system. The gold standard for bacterial identification used in this study was DNA sequencing of 16S rDNA (ribosomal subunit). Chromogenic agar (96%) and MALDI-TOF (95%) identified a significantly greater number of bacterial isolates as compared with the BBL Crystal (86%) and API identification (38%) systems. The BBL Crystal system correctly identified a significantly great number of Gram-negative isolates (93% accurate) as compared with *S. zooepidemicus* isolates (75% accurate). In conclusion, MALDI-TOF and chromogenic agar were able to precisely identify both Gram-positive and Gram-negative equine uterine pathogens. Matrix-assisted laser desorption/ionization–time of flight systems are currently used in veterinary diagnostic laboratories; however, chromogenic agar can be incorporated into clinical practice to rapidly and accurately identify common equine uterine pathogens.

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1. Introduction

Endometritis is a significant cause of reduced pregnancy rates in equine breeding programs resulting in substantial economic loss every year [1]. Bacterial endometritis is associated most commonly with isolates of *Streptococcus equi* spp. *zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [2,3]. A diagnosis of bacterial endometritis is based on a combination of reproductive history, clinical signs, physical examination, and the results of specific diagnostic tests [4–6]. Veterinarians rely on accurate genus and species identification of bacteria cultured from the equine reproductive tract first to determine if an organism is pathogenic and secondly in developing a therapeutic plan [5,6].

A variety of laboratory procedures are available to determine the identification of bacterial genus and species. Historically, methods to determine bacterial identification required time intensive procedures such as evaluation of

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colony morphology, microscopic cellular characteristics, and finally multiple individual physiological or biochemical tests. These laborious physiological and biochemical tests have been commercialized into a panel in which multiple phenotypic assays are performed concurrently and the panel of results are compared with a known library to determine bacterial identification [7–9]. Chromogenic agar has been developed for high-throughput screening of pathogenic bacteria of interest to the microbiology laboratory [10–12]. Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) determines bacterial identification through detection of bacterial proteins and the resulting protein mass spectrum is compared with a compilation of known bacterial spectral patterns [13,14]. The current gold standard for bacterial identification is DNA sequencing of the 16S ribosomal subunit as compared with a library of known DNA sequences [15].

The aim of this study was to compare the ability of four different methods of bacterial identification to accurately identify the four major equine uterine pathogens, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. equi* spp. *zooepidemicus*.

2. Materials and Methods

Equine uterine isolates of *S. equi* spp. *zooepidemicus* (n = 25), *E. coli* (n = 25), *P. aeruginosa* (n = 25), and *K. pneumoniae* (n = 25) were selected randomly from a pool of organisms isolated from the equine uterus stored in glycerol at −80°C [16]. The 100 isolates evaluated in the study were randomly assigned an identifier from 1 to 100. The freezer stock was streaked onto TSA plates with 5% sheep blood and incubated at 37°C for 18 hours. The plates were observed for the presence/absence of pure microbial growth for allocation to the four methods to determine bacterial identification. Personnel performing and interpreting the assays had no knowledge of the identity of the bacterial strains in the study.

2.1. 16S rDNA Sequencing

For all 100 bacterial strains, a sterile DNA-free pipette tip was used to pick up two single bacterial colonies from the agar were placed in 0.5 mL bacterial DNA-free water (UltraClean PCR Water, Mo Bio, Carlsbad, CA) in a DNA-free microcentrifuge tube and placed in a −80°C freezer. Samples were thawed, DNA extracted (DNeasy Blood and Tissue Kit, Qiagen, Germantown, MD), and the bacterial 16S rDNA (ribosomal subunit) was amplified. The amplification process was performed in a final volume of 25 μL containing 12.5 μL of commercial fluorescent PCR dye (Q5 High-Fidelity 2X Master Mix, New England Biolabs, Ipswich, MA), 1 μL of 27F and 1 μL of 1492R primers [17], 5 μL of extracted template DNA, 2 μL of dimethyl sullfoxide, and 3.5 μL of sterile DNA free water (UltraClean PCR Water, Mo Bio, Carlsbad, CA). All PCR reactions were performed on a thermocycler (Mastercycler nexus, Eppendorf, Hamburg, Germany) with the following conditions: 5 minutes at 95°C, followed by 40 cycles (each cycle consisted of a denaturation step [10 seconds at 95°C], followed by an annealing step [35 seconds at 60°C]). DNA was submitted to a commercial laboratory for sequence analysis. Chromatograms were analyzed and results were compared with published sequences using the BLAST sequence similarity tool (megaBLAST algorithm, accessed via NCBI: www.ncbi.nlm.nih.gov) using default settings. 16S ribosomal subunit sequencing is the gold standard for bacterial identification, and all other assays evaluated in the present study were compared with the results obtained by this method [17–25].

2.2. MALDI-TOF

The biotyper plate (Bruker Daltonics, Inc) was prepared by adding 1 μL of 70% formic acid to each selected sample spot. A single isolated colony of the unknown bacterium was selected and mixed with the formic acid, in triplicate. The smear was left to dry at room temperature, followed by addition of 1 μL of α-cyano-4-hydroxycinnamic acid (α-matrix). The biotyper plate was loaded into the MALDI-TOF platform (MALDI Biotyper, Bruker Daltonics, Billerica, MA), spectral scans obtained, and compared with the known library of spectral scans (BDAL Library, MALDI Biotyper 3.1, Bruker Daltonics, Billerica, MA). Every MALDI-TOF run included a sample consisting of a bacterial test standard (Bruker Daltonics, Billerica, MA) to serve as a positive control.

2.3. Gram Stain

Classification of bacterial samples as Gram-positive or Gram-negative is required before using the BBL Crystal Identification System and API Identification Strips as a different panel of phenotypic tests are used for either Gram-positive or Gram-negative bacteria. Gram stains were performed on an isolated colony from the agar plate, as previously described [26]. If Gram-negative bacteria were identified, supplemental oxidase and indole tests (BBL DrySlide Oxidase and BBL DrySlide Indole, Becton, Dickinson, and Company, Franklin Lakes, NJ) were performed. To detect the presence of oxidase, an isolated colony was smeared onto the reaction area. If the reaction area turned dark purple within 20 seconds, then it was considered a positive reaction for the detection of oxidase. The same procedure was performed for indole testing. If the reaction area changed from yellow to pink within 30 seconds, then it was considered a positive identification of indole production.

2.4. BBL Crystal Identification System

The BBL Crystal Identification System was performed per the manufacturer's instructions. Briefly, a single bacterial colony was dispersed into a tube of inoculum fluid. The tube was capped and vortexed for 10–15 seconds. The turbidity of the inoculum was adjusted to a 0.5 McFarland standard. The inoculum fluid was poured into the target area of the base so that all wells were filled with inoculum fluid. Based on results of the Gram stain, Gram-positive bacteria were evaluated with BBL Crystal Gram-positive ID kit (BBL Crystal GP) and Gram-negative bacteria with BBL Crystal Enteric/Nonfermenter ID kit (BBL Crystal E/NF). The panel was incubated, label side down,