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Morphological, biochemical, and antimicrobial susceptibility characterization: A comparative study of *B. abortus* S19 and *B. abortus* S19Δ*per*

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Abstract
Brucellosis is a contagious and under-reported zoonotic disease of livestock, wild animals, and humans. As a result, the livestock economy was severely affected worldwide, including in India. To date, *Brucella abortus* S19 strain-based vaccine is available in India to control the disease. However, the S19 vaccine strain also has disadvantages of the virulent nature. *B. abortus* S19 Δ*per* is a mutant vaccine candidate created to overcome S19 strain disadvantages. While developing S19Δ*per* candidate, many other strains of *Brucella* cultures were used for vaccine efficacy testing in an animal model. All laboratory-maintained pathogenic cultures were known to cause infection in humans. This overall concept prompted us to characterize the morphological, biochemical, and antimicrobial susceptibility of newly developed mutant strain along with pathogenic cultures of *B. abortus* and *B. melitensis*. In the initial study, we characterized the morphology, biochemical, and growth rate of *B. abortus* S19 and *B. abortus* S19Δ*per* strains. Further, we tested the antimicrobial susceptibility of *B. abortus* S19 and *B. abortus* S19Δ*per*, along with other standard pathogenic cultures. Commonly recommended antibiotics for treatments were used to assess the *in vitro* antimicrobial susceptibility testing (AST). The morphological and biochemical characterization results showed no significant difference between the S19 and S19Δ*per*. *Brucella* strains showed *in vitro* susceptibility to tetracycline, levofloxacin, streptomycin, kanamycin, ampicillin, imipenem, amikacin, ceftriaxone, trimethoprim/sulfamethoxazole, and azithromycin. Vaccine strains were susceptible to penicillin and ampicillin. Further, MIC strips of cell wall antibiotics were used, and varying degree of resistance pattern was noticed among polymyxin, colistin, penicillin, and vancomycin to *B. abortus* S19 and *B. abortus* S19Δ*per*. In conclusion, the sensitivity of commonly used drugs for treating Laboratory associated brucellosis should be regularly monitored. To minimize future drug resistance, we propose rationalizing the use of antibiotics in selective culture media.

Keywords: Antimicrobial resistance, *Brucella abortus* S19, MIC, *B. abortus* S19 Δ*per*

Introduction
Brucellosis is a contagious and economically significant disease for livestock health. It is one of the re-emerging and neglected zoonotic diseases worldwide. Livestock plays the most crucial role in the Indian economy, and India holds the world's largest livestock population (538 million per the 20th livestock census). Brucellosis is endemic to both livestock and humans in India (DAHD, 2012). In livestock, it causes reproductive failures that lead to economic losses, while it causes more chronic febrile infections in humans (Lindahl *et al*., 2019; Peng *et al*., 2020) [15, 21]. Brucella is a Gram-negative coccobacillus pathogen that affects cattle (*B. abortus*), sheep and goats (*B. melitensis*), pigs (*B. suis*), rams (*B. ovis*), dogs (*B. canis*), wild rodents (*B. neotomae*), bison, elk, camels, deer, buffalo and marine species. However, cross-infection with *Brucella* has been confirmed between animal species (Saddique *et al*., 2019). Brucellosis can be controlled with a proper vaccination program. Vaccine strains such as *B. abortus* S19 as well as *B. abortus* RB51 are used in many countries, even though these strains have their advantages and disadvantages. *B. abortus* S19 Δ*per* vaccine strain developed with modified lipopolysaccharide (LPS) structure. This strain has been created with the aim of safety, residual virulence, and DIVA capability. This vaccine strain protects against Brucella infection equally as S19 strain (Lalsiamthara *et al*., 2015; Chaudhuri *et al*., 2021) [14, 5]. Pathogenic strains such as *B. abortus* 544, *B. melitensis* 16M and *B. abortus* S99 were maintained in the Laboratory for challenge study and production of diagnostics.
Laboratory-associated *Brucella* infection is higher than other diseases. It can be readily aerosolized and infect persons with a minimum of 100-1000 bacteria (Pappas *et al*., 2006) [20].

Antibiotic therapy can cure diseases with various complications, and relapse occurs most of the time. The commonly suggested therapy for brucellosis is triple antibiotics doxycycline and streptomycin with Rifampin (Bayindir *et al*., 2003) [2]. In laboratory settings, *Brucella* strains are cultivated under the selective pressure of antibiotics such as penicillin, vancomycin, Nystatin, and colistin. Analyzing the antibiotic resistance patterns of laboratory-maintained cultures could help to suggest treatment and differentiate the vaccine and pathogenic strains. With its theme, morphology, biochemical, and antibiotic sensitivity patterns of the lab-maintained isolates were analyzed, and antibiotics can differentiate these strains could be identified. This study aimed to characterize laboratory-maintained cultures' complete morphology and biochemical and antimicrobial sensitivity, including standard vaccine and pathogenic strains.

Materials and Methods

Revival and Maintenance of bacterial cultures

All *Brucella* cultures (n= 5) were revived using Brucella broth (Himedia, India) for 48-72 hours at 37 °C. *B. abortus* 544 was incubated with 5% CO_2, but other cultures were incubated in the regular bacteriological incubator. A loopful of culture from Brucella broth streaked onto the Brucella agar and Potato infusion agar (Himedia, India) plates and incubated at 37 °C for 48-72 hours to confirm the colony characteristics. Subsequently, cultures were streaked into the MacConkey agar (Himedia, India) plates to confirm the purity of the cultures. The cultures were maintained in the Brucella agar plates throughout the study.

Morphological characterization

Colony characters such as color, transparency, margin, and size of colonies were examined and compared according to Alton, 1988 and OIE, 2022. In addition, the microscopic structure of bacterial cells was examined for all isolates with gram staining protocol.

Smooth and rough characterization of colonies were examined by using crystal violet and acriflavine dye.

Crystal violet dye method (White and Wilson method)

Freshly prepared 2% crystal violet stain flooded over the Brucella colonies on the agar plates for 30 secs. Excess stains from the plates were removed by ???, and the color of the colonies were examined (reference).

Acriflavine Dye agglutination test (Brun and Bonestell method)

Brucella colonies were picked up from agar plates, re-suspended in 10µl normal saline solution, and mixed with 10µl of 0.1% acriflavine aqueous solution. The rate of agglutination was examined and recorded macroscopic and microscopically (40x) (Alton, 1988).

Biochemical Characterization

Catalase test

A loopful of colonies were mixed with 3% hydrogen peroxide solution and formation of gas bubbles was considered as positive for catalase production.

Oxidase test

Bacterial colonies were picked up from the *Brucella* agar plates smeared on the readymade oxidase disk (Himedia, India). Positive test was recorded as the blue color development within 10-15s.

Urease test

Christensen's medium was used for the urease test. A loopful of colonies were inoculated in the urea broth and then incubated at 37 °C for 24 hours. Development of pink color generation indicated positive for urease test.

Nitrate reduction test

The single colony of *Brucella* from Brucella agar plates was inoculated into the tryptone broth with nitrate disc and then incubated at 37 °C for 48-72 hours. Addition of 2-3 drops of sulphanilic acid and N, N- Dimethyl-1-Napthylamine reagent to the tryptone broth changes color to red, indicating a positive test.

Hydrogen sulfide (H_2S) production test

The Brucella-grown broth was utilized for the test. A lead acetate strip was kept hanged into the test tubes and incubated at 37 °C for 24 hours. The positive test indicated by the development of black color in the strips.

Antimicrobial susceptibility testing

E-test strip method

In vitro evaluation of membrane attacking antibiotics efficacy against *B. abortus* S19 and *B. abortus* S19 Δ*per* were measured with minimum inhibitory concentration (MIC) values. Ezy strips (Himedia, India) of Polymyxin B, Colistin, Penicillin, and Vancomycin were used to identify the MIC values. The test was performed by using 0.5 McFarland of culture in Muller Hinton broth supplemented with 5% calf serum.

Disc diffusion method

Antibiotic susceptibility test (Kirby Bauer method) for Brucella cultures with antibiotics such as Rifampin, doxycycline, streptomycin, colistin, polymyxin, vancomycin, penicillin, azithromycin, ampicillin, kanamycin, amikacin, levofloxacin and sulfa-trimethoprim were used to identify the susceptability pattern. Muller Hinton agar with 5% calf serum was used to culture the bacteria, and CLSI guidelines breakpoints for *Haemophilus* sp. were used to measure and compare the resistance diameter (CLSI, 2020; Kosikowska *et al*., 2020) [13].

Results and Discussion

Brucellosis is a serious public health issue in India. It is causing a significant impact on the economic status of livestock-associated industries. Brucellosis in livestock is responsible for a median loss of US $ 3.4 billion. *Brucella* organisms localize in the secretory organs and excrete through milk and uterine discharges. Transmission of *Brucella* occurs through contact with contaminated materials and ingesting livestock-associated products (Cordes and Carter, 1979) [6].

Humans, particularly veterinarians and laboratory workers associated with *Brucella* handling in labs, are more prone to occupational infection. (Luna Martinez and Mejia-Teran, 2002) [18]. The *Brucella* strains such as *B. abortus* S19 and *B. abortus* 544, S99, and *B. melitensis* 16M are the most common Laboratory maintained standard strains. These strains can potentially infect humans and secrete from

livestock secretions. A new vaccine candidate *B. abortus* S19 Δ*per* developed from the *B. abortus* S19 vaccine strain. It is having *wbk*B gene deletion from the S19 strain, which expresses smooth intermediate characters of lipopolysaccharides. This strain has many safety and virulence attenuation advantages over the existing vaccine strain and may have DIVA capacity (Lalsiamthara *et al*., 2015) [14].

Morphological characterization of *Brucella* strains

B. abortus S19 and *B. abortus* S19Δ*per* colonies were round, smooth, and pale greyish white. It required 48-72 hours for appreciable colonies to grow on Brucella agar plates, incubated at 37 ºC, and does not require CO_2 supplementation. *B. abortus* 544 and *B. melitensis* 16M colonies were round, little mucoid with smooth margins. *B. abortus* 544 strains supplemented with 5% CO_2. They are translucent and light honey colour when viewed through a transparent medium. Colonies appeared pearly white and convex when viewed from above. Colonies were observed as enlarged and gradually darkened over time. Gram staining and microscopic examination of *Brucella* cultures revealed all the organisms were gram-negative (pink/red) and coccobacilli in structure (Fig No 1).

Smooth and rough differentiation of colonies was observed with the crystal violet and acriflavine dye agglutination test. Smooth strains cannot stain with crystal violet and also not form any agglutination with acriflavine dye (OIE, 2022). *B. abortus* S19 and *B. abortus* S19 Δ*per* behaving similarly, and both strains were not stained with crystal violet dye. No visible agglutination was noticed in the acriflavine dye test. Microscopic observation (10x and 40x) of the acriflavine dye test indicated mild agglutination in the *B. abortus* S19Δ*per* strain (Figure No 2). All other reference strains do not take up the dye and indicates smooth nature of colonies.

Fig 1: Morphological characterization of *Brucella* cultures

B. abortus S19 and *B. abortus* S19 Δ*per* colonies. Visualization of *Brucella* colonies under a stereomicroscope (40x)

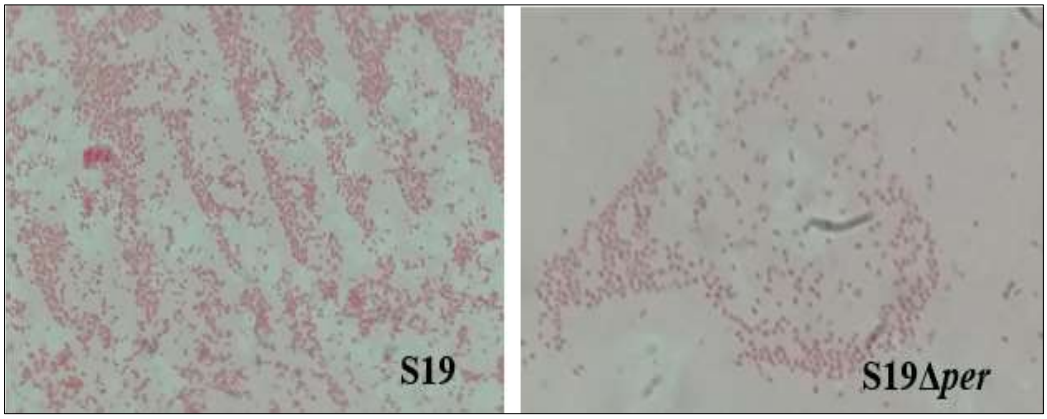

Fig 2: Gram staining of *Brucella* cultures

Gram staining image of *B. abortus* S19 and *B. abortus* S19 Δ*per* culture under light microscope, Oil immersion (100x).

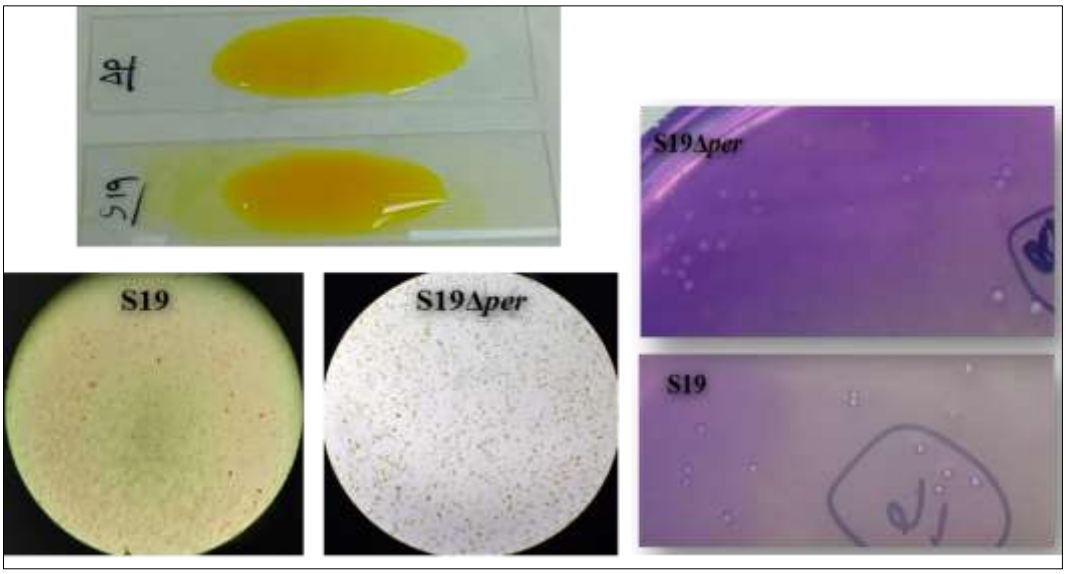

Fig 3: Crystal violet and Acriflavine dye test of *Brucella* cultures

Smooth, rough characterization of *Brucella* colonies with crystal violet and acriflavine agglutination test. Macroscopic observation of colonies and agglutination (OIE manual, 2022).

Biochemical characterization of *Brucella* strains

The biochemistry profiles such as oxidase, catalase, urease, and nitrate reduction test were positive in all the *Brucella* strains (White and wilson, 1951). All the *B. abortus* strains produced H_2S positive and *B. melitensis* 16M showed negative for H_2S production test (Figure No 3).

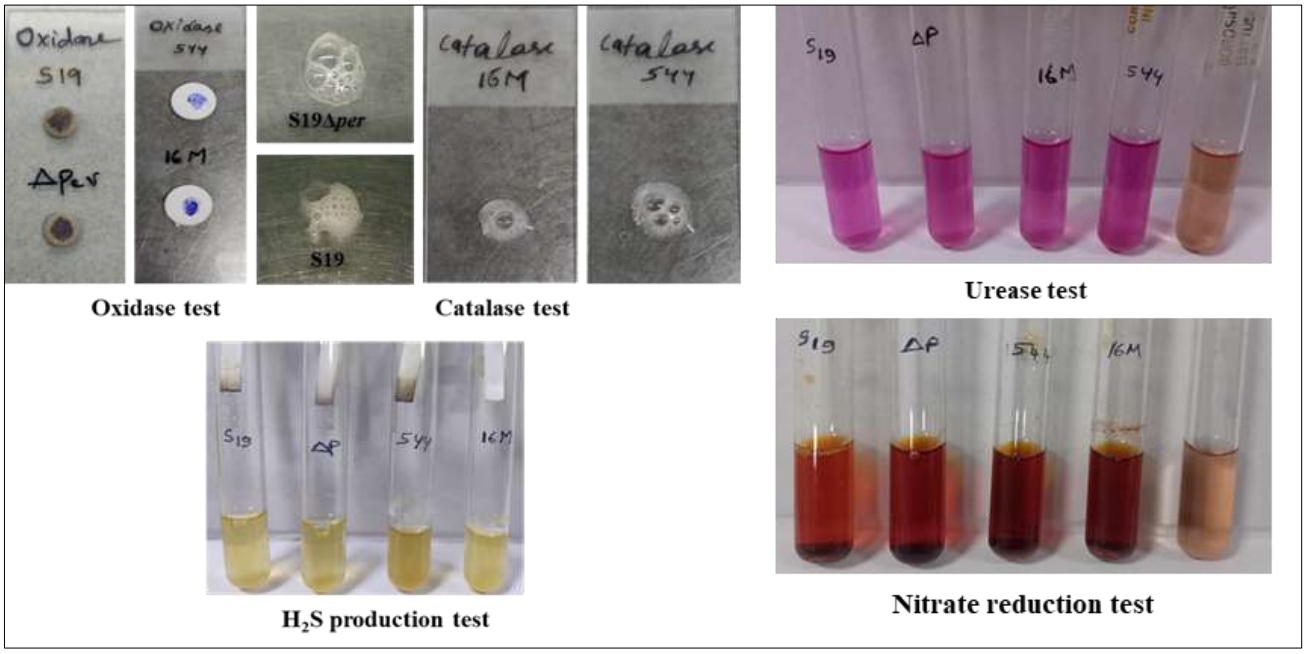

Fig 3: Catalase, Oxidase, H_2S production test, Urease, and nitrate reduction test for *Brucella* strains

Antimicrobial sensitivity analysis of *Brucella* strains

Antibiotics recommended for *Brucella* treatments were used for the AST assay. All the *Brucella* cultures, including *B. abortus* S99 were tested for antibiotic susceptibility. Each culture's sensitivity varied depending on its virulence nature. *B. abortus* S19 and *B. abortus* S19 Δ*per* vaccine strains subjected to the Antibiotic sensitivity test with MIC strips (Figure No 4). Among that, *B. abortus* S19 Δ*per* strains were showed less MIC value to the antibiotics such as polymyxin B, penicillin, and colistin but resistant to vancomycin antibiotics (Table No 2). *B. abortus* S19Δ*per* membrane modification allows polymyxin B to bind and increased sensitivity. Cationic antibiotics bind with negative charge of bacterial membrane especially lipidA and destroy the bacteria. It suggested that increased *B. abortus* S19Δ*per* surface to cationic antibiotics (Ayoub Moubareck, C., 2020) [31].

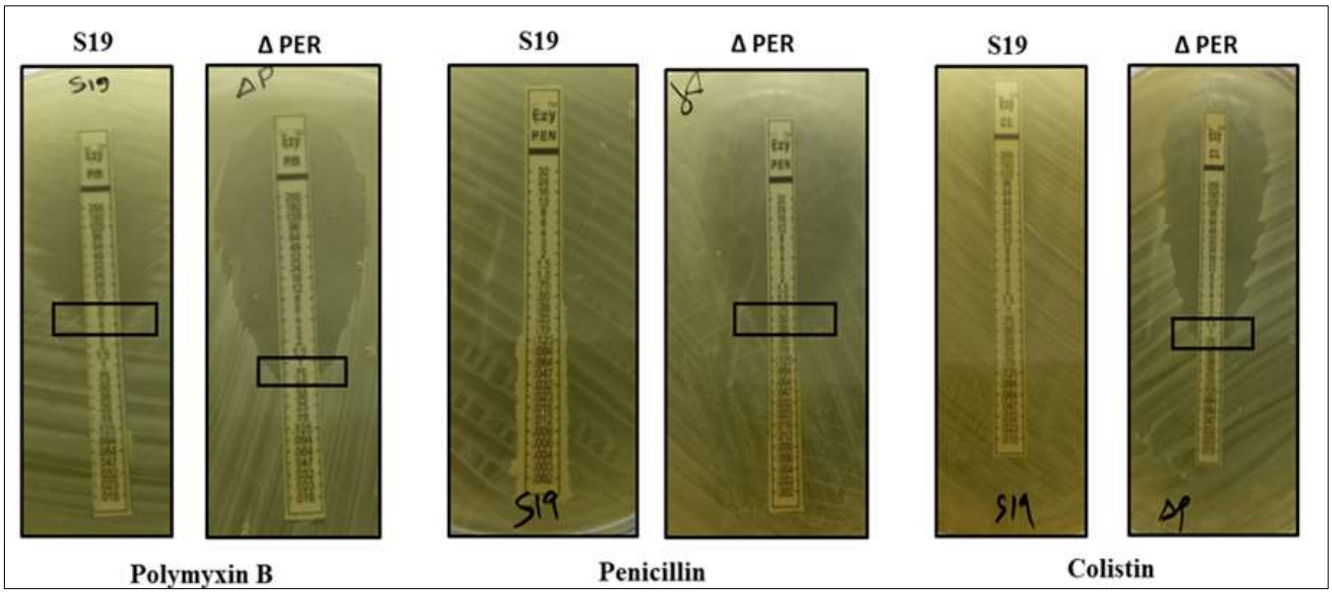

Fig 4: MIC analysis of polymyxin B, Penicillin, and colistin for *Brucella* strains

Table 1: Details of Antibiotics, *Brucella* strains and MIC values

S. No	Antibiotics	*B. abortus* S19	*B. abortus* S19 Δ*per*
1.	Polymyxin B	4 mcg/mL	0.75 mcg/mL
2.	Penicillin	Resistance	0.50 mcg/mL
3.	Colistin	Resistance	0.75 mcg/mL
4.	Vancomycin	Resistance	Resistance

B. abortus S19 Δ*per* showed resistance against kanamycin antibiotic but all other *Brucella* isolates were sensitive. Penicillin inhibits the growth of *B. abortus* S19 and *B. abortus* S19 Δ*per* strain but no other *Brucella* isolates. Vancomycin inhibits the *B. melitensis* strains, not *B. abortus* strains. *B. abortus* strain 544 showed resistance to azithromycin were as other isolates were sensitive. *B. melitensis* 16M showed resistance to polymyxin; intermediate resistance was noticed among other *B. abortus* isolates. *B. abortus* isolates showed intermediate resistance to Rifampin but *B. melitensis* 16M strain was sensitive to the antibiotic. All the isolates showed resistance to colistin and highly sensitive to sulfamethoxazole-trimethoprim, ceftriaxone, streptomycin, amikacin, tetracycline, imipenem, and levofloxacin. (Table No: 3). Many field isolates from India and other countries were developing antimicrobial resistance against commonly used antibiotics for the treatment (Baykam *et al*., 2004; Dojmari *et al*., 2018) [3, 7]. *Brucella* strains circulating in Kazhakastan and Iran showed intermediate resistance towards to Rifampin which commonly used in the treatment protocol. (Shevtsov *et al*., 2017; Alamin *et al*., 2019) [28, 29]. Sulfamethoxazole-trimethoprim resistance was seen in *Brucella* isolates from northeastern Europe, while isolates included in our study were sensitive (Arapović *et al*., 2022) [30].

However, laboratory-maintained cultures developing antimicrobial resistance against antibiotics were not analyzed frequently. Rifampin resistance was noticed among field isolates commonly, and it is included in the treatment regimen. Antimicrobial susceptibility of membrane attacking antibiotics could change based on the lipopolysaccharides expression. Hence, LPS-altered strain S19Δ*per* more susceptible to cell wall-attacking antibiotics (Sabins *et al*., 2021) [22].

Table 2: Sensitivity pattern of *Brucella* cultures with recommended antibiotics

Antibiotics disc	S19	S19Δ*per*	544	S99	16M
Levofloxacin	S	S	S	S	S
Kanamycin	S	R	S	S	S
Polymyxin	I	I	I	I	R
Ampicillin	S	S	R	R	R
Imipenam	S	S	S	S	S
Tetracycline	S	S	S	S	S
Vancomycin	R	R	R	R	S
Amikacin	S	S	S	S	S
Peniciliin	S	S	R	R	R
Streptomycin	S	S	S	S	S
Ceftriaxone	S	S	S	S	S
Azithromycin	S	S	R	S	S
Rifampin	I	I	I	I	S
Colistin	R	I	R	R	R
Sulfa-trimethoprim	S	S	S	S	S

Conclusion

The study envisaged characterizing different *Brucella* cultures, including vaccine candidates. The Antimicrobial sensitivity and resistance pattern of *Brucella* cultures elucidated. Antibiotics such as kanamycin, ampicillin, penicillin, vancomycin, and polymyxin B can be employed to allow the differential growth of *Brucella* cultures. MIC analysis of penicillin, polymyxin B, and colistin shows the difference in *B. abortus* S19 and *B. abortus* S19Δ*per* strain.

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