Acta Tropica 172 (2017) 70–74

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Isolation, characterization, virulence and immunogenicity testing of field isolates of *Pasteurella multocida*, *Staphylococcus aureus*, and *Streptococcus agalactiae* in laboratory settings

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ARTICLE INFO

Keywords:
P. multocida
S. aureus
Str. agalactiae
Pathogenicity
Immunogenicity
Montanide®

ABSTRACT

The present study was designed to investigate isolation, characterization, virulence and immunogenicity testing of field isolates of *Pasteurella multocida*, *Staphylococcus aureus*, *and Streptococcus agalactiae* in rabbits and mice. Isolates of *P. multocida*, *S. aureus* and *Str. agalactiae* recovered from field cases of Hemorragic septicemia and mastitis were scrutinized for virulence/pathogenicity and immunogenicity. Mouse LD_{50} of *P. multocida* showed that *P. multocida* isolate No.1 was more virulent than isolates No. 2 and 3. Virulence of isolate No.1 *S. aureus* and *Str. agalactiae* revealed that 100, 80% rabbits died within 18 h of inoculation. Seven-digit numerical profiles of these 4 isolates with API® Staph test strips isolates, No.1 (6736153) showed good identification (*S. aureus* id = 90.3%). Indirect ELISA-based serum antibody titers to *P. multocida* isolate No.1, *S. aureus* No.1, *Str. agalactiae*, isolate No.1 elicited high antibody titers 1.9, 1.23, 1.12 respectively.

Conclusion: All the pathogens of Isolate No. 1 (*P. multocida, S. aureus Str. agalactiae)*, were high antibody than others isolates.

1. Introduction

Hemorrhagic septicemia (HS) and mastitis are the two most prevalent and economically important diseases of dairy animals in Pakistan (Khan et al., 1994–1995; Ali et al., 2016). Vaccination using different types of vaccines (e.g. formalin-killed alum precipitated bacterin, oil adjuvanted vaccines) is the single most important control measure against HS in Pakistan and in other Asian, African, South European and Middle East countries where this disease is endemic (Benkirane and De Alwis, 2002; Sotoodehnia et al., 2005; Zamri-Saad and Annas, 2016). Whereas, vaccination against HS is practiced in routine, the application of mastitis control measures is almost totally nonexistent in Pakistan, save a small number of large commercial dairy herds. A combined HS-mastitis vaccine, therefore holds the promise of a simultaneous control of these two rife dairy animal diseases in Pakistan.

Staphylococcus aureus and *Str. agalactiae* are the most prevalent etiologic agents of mastitis in dairy animals in Pakistan (Allore, 1993; Memon et al., 1999; Sharif and Muhammad, 2009). Over the past several years, researchers at the Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad have demonstrated that a cost-effective control of mastitis is an attainable dairy health objective with the use of locally prepared mastitis vaccines containing these two mastitis pathogens (Athar, 2007; Ahmad and Muhammad, 2009). Evaluation in a laboratory setting is the first step in the development of a new vaccine (Anon, 2012). Characterization of candidate vaccinal isolates in terms of biochemical and other features as well as virulence and immunogenicity for preparing a vaccine for filed use.

The present study was planned for isolation, characterize, virulence and immunogenicity is a prerequisite testing of filed isolates of *P. multocida*, S. *aureus*, *and Str. agalactiae* in laboratory settings.

2. Materials and methods

2.1. Isolation, and characterization isolates of P. multocida, S. aureus and Str. agalactiae

Peripheral blood samples of 5 buffalo calves and 2 adult buffaloes suffering from HS, bone marrow from femurs of 4 buffaloes that had died of HS during the past 48–72 h and heart blood of 4 additional buffalo calves immediately after their slaughtering prompted by their moribund condition with overt clinical HS (De Alwis, 1992) were collected aseptically and processed for the isolation of *P. multocida* as per Anon (2012). The samples were cultured on casein-sucrose-yeast (CSY) agar and blood agar plates. Further confirmation of the organism

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http://dx.doi.org/10.1016/j.actatropica.2017.04.020
Received 11 January 2017; Received in revised form 20 April 2017; Accepted 21 April 2017
Available online 24 April 2017

and its type (i.e., type B) was done through biochemical reactions (Ekundayo et al., 2008; Ashraf et al., 2011) and by using PCR primers (Anon, 2012).

For the isolation of *S. aureus* and *Str. agalactiae* isolates, milk samples from 37 clinically mastitic quarters (per acute or acute) of cows (n = 10) and buffaloes (n = 9) were collected aseptically following the procedure described by Hogan et al. (1999). Each milk sample was inoculated onto a blood agar plate containing 5% defibrinated sheep blood and MacConkey's agar plate. Gram positive, catalase positive coccal isolates that grew on blood agar plates were presumptively identified as staphylococci and subjected to tube coagulase test (Hogan et al., 1999). Staphylococcal isolates showing a positive tube coagulase test after 4 h incubation at 37 °C were identified as *S. aureus* and biotyped using commercially available kit of the genus Staphylococcus (API® Staph. bioMerieux, France). Latex Slide Agglutination test was performed using Staphytect Plus® kit (Oxoid Ltd, Basingstoke, UK) for the determination of clumping factor, protein A and certain polysaccharides found only in *S. aureus* (Rashid, 2011; Raza, 2011). The definitive identification of *S. aureus* was done by using PCR primers (Brakstad et al., 1992). Quantitative determination of biofilm production trait of *S. aureus* isolates was undertaken sepectrophotometerically on a flat bottom 96 well ELISA plate and qualitative determination by tube method and Congo red agar method modified from Mathur et al. (2006) by Rashid (2011). Gram positive, catalase-negative, esculin-negative, CAMP- positive, and β-hemolytic coccal isolates (n = 2) were considered as isolates of *Str. agalactiae* (Altwegg and Bockemühl, 1998; Hogan et al., 1999) and biotyped using a commercially available kit of genus Streptococcus (API® 20-Strep., bioMerieux, France).

2.2. Determination of virulence P. multocida, S. aureus and str. agalactiae isolates

Virulence (LD_{50}) of candidate vaccinal *P. multocida* isolates (n = 3) was determined in 6 weeks old Swiss albino mice (n = 50), as per Butt et al. (2003) and Naz et al. (2012). Mortality and time of death associated with each tenfold dilution (10^{-1}–10^{-10} in sterile normal saline) were recorded to calculate LD_{50}. Virulence of each isolate of *S. aureus* (n = 4) and *Str. agalactiae (n = 2)* was determined in 15 adult rabbits by subcutaneous inoculation of 0.2 ml of bacterial suspensions (1×10^{10} cells/ml) grown in nutrient broth. The inoculated rabbits were observed for up to 48 h post inoculation for mortality (Ahmad, 2009). Dead rabbits were subjected to necropsy examination

2.3. Immunogenicity testing of P. multocida, S. aureus and Str. agalactiae isolates

Immunogenic response of candidate vaccinal isolates (*P. multocida*, n = 3; *S. aureus* n = 4 and *Str. agalactiae* n = 2) was determined in rabbits (n = 36). Each of these candidate vaccinal isolate was inoculated (0.2 ml formalin killed suspensions containing 1×10^6 per ml) subcutaneously twice at an interval of 7 days (Athar, 2007). Serum antibody titers were determined by an in-house indirect ELISA.

3. Results

3.1. Isolation, biochemical reactions and identification of P. multocida, S. aureus and str. agalactiae isolates

Microbiological examination of peripheral blood, femur, and heart blood of HS affected animals on CSY and blood agar plates yielded growth of only three suspect isolates of *P. multocida*. No *P. multocida* isolate was recovered from peripheral blood and swab samples of heart blood. Culturing of heart blood and femur bone marrow samples respectively yielded growth of two and one isolate of *P. multocida*. Suspected isolates of *P. multocida* formed smooth grayish, glistening translucent colonies of nearly 1 mm diameter on blood agar after 24 h incubation at 37 °C. On Gram staining, the organism appeared as Gram −ve, short, ovoid bipolar rods with some degree of pleomorphism. Growth on CYS agar was smooth, yellowish, mucoid and larger in size than that on blood agar. All three *P. multocida* isolates were positive for indole, ODC, glucose, fructose, catalase and maltose and negative for H_2S production, D-dulcitol, and lactose. Variable results were recorded for D-sorbitol and arabinose (Table 1). Isolates of *P. multocida* were confirmed by amplification of *P. multocida* specific gene using KTSP61/KTT7 set of primers. These primers yielded a product of 618 bp on electrophoresis which confirmed these isolates as *P. multocida* type B.

Table 1
Biochemical profiles of *P. multocida* isolates (n = 3) recovered from femur bone marrow and heart blood samples of HS affected animals.

Biochemical test	No. of *P. multocida* isolates showing +ve or −ve reaction
Indole	All three isolates showed a +ve reaction
ODC[a]	All three isolates showed a +ve reaction
H_2S on TSI[b]	All three isolates showed a −ve reaction
D-sorbitol	Two isolates showed +ve reaction and one isolate showed −ve reaction
D-dulcitol	All three isolates showed a −ve reaction
Arabinose	One isolate showed +ve reaction and two isolates showed-ve reaction
Glucose	All three isolates showed a +ve reaction
Fructose	All three isolates showed a +ve reaction
Lactose	All three isolates showed a −ve reaction
Catalase	All three isolates showed a +ve reaction
Maltose	All three isolates showed a +ve reaction

[a] ODC = Ornithine decarboxylase activity.
[b] TSI = Triple Sugar Iron agar.

Microbiological examination of 37 clinically mastitic (peracute/actue) quarters of cows (n = 10) and buffaloes (n = 9) on blood agar and MacConkey's agar plates yielded growth of 10 suspect *S. aureus* and 2 suspect *Str. agalactiae* isolates. These isolates grew only on blood agar and there was no growth on MacConkey's agar plates. *Staphylococcus aureus* isolates (n = 10) that grew on blood agar were β- hemolytic, catalase positive, Gram positive cocci and were thus presumptively identified as members of the genus *Staphylococcus*. On performing tube coagulase test, 4 of the 10 isolates tested positive for coagulase production at 4 h. On blood agar, suspect *Str. agalactiae* isolates (n = 2) displayed β-hemolysis. Microscopic examination of Gram stained smears of these isolates revealed Gram positive cocci. On catalase test, these isolates were negative. CAMP test reaction on blood agar with 0.1% esculin and 0.01% ferric citrate was positive for both the isolates. Seven-digit numerical profiles of these 4 isolates with API® Staph (bioMerieux, France) test strips, among these, isolate number 1 (6736153) showed good identification (*S. aureus* id = 90.3%). All 4 isolates produced biofilms, however, the highest OD (0.31 nm) was noted in isolate # 1, OD values ranged between 0.17- 0.22. Thus, the isolate #1 was considered as the strongest producer of biofilm with spectrophotometric method. Tube method-based scores of biofilm production by 4 candidate vaccinal *S. aureus* isolates indicated that isolate number 2, 3 and, 4 showed a moderate level of biofilm production, whereas isolate number 1 showed strong biofilm production. Isolate number 2, 3, and 4 showed intermediate degree of biofilm production, whereas isolate number 1 showed strong degree of biofilm production on Congo red agar (CRA). Biotypes 2 candidate vaccinal *Str. agalactiae isolates* in terms of seven-digit numerical profiles determined by using a commercially available kit API® 20 STREP, bioMérieux, France) as shown in Table 2. Among these, isolates number 1 (3462414) showed very good identification (*Str. agalactiae* % id = 99.9). These isolates were also confirmed as *S. aureus* as they displayed extracellular thermostable nuclease (nuc) activity in PCR by yielding a 279 bp product.