Serotyping of *Pasteurella multocida* and *Mannheimia haemolytica* Isolates from Pneumonic Sheep in The Sudan Using the Indirect Haemagglutination Test

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ملخص البحث

أجريت هذه الدراسة فى معهد أندراستيبورت البيطرى (OVI) بجنوب افريقيا لمعرفة الأنماط المصلية لثلاث معزولات من المانهيمية الحالة للدم (*Mannheimia haemolytica*) وثلاث معزولات من الباستيرية الفاتكة (*Pasteurella multocida*). عزلت هذه المعزولات من رئات ضان ملتهبة جمعت من سلخانة الدمازين بولاية النيل الازرق.تم التصنيف بواسطة اختبار التراص الدموى غير المباشر حسب الخطوات المتبعة للتصنيف بالمعهد . تم توفير امصال الضد المرجعية للمانهيمية الحالة للدم و الباستيرية الفاتكة من معهد اندراستيبورت. استخدمت عترتي الباستيرية الفاتكة ذوات النمط المصلى A (ATCC=12945) و النمط المصلي D(ATCC=12948)وعترة المانهيمية الحالة للدم ذات النمط المصلي A (ATCC=4270s) كعترات تحكم ومقارنة . كانت نتائج التصنيف للباستيرية الفاتكة هى 3: A_1 , 3 :A_1 , 3 : A_2 والمانهيميا الحالة للدم هى A1, A2, A2 .

Summary

This study was conducted at the Onderstepoort Veterinary Institute (OVI) to serotype *Mannheimia haemolytica* and *Pasteurella multocida* isolates from pneumonic lungs of sheep. Six isolates, three of each *Mannheimia haemolytica* and *Pasteurella multocida* from El-Damazin area, Blue Nile State, the Sudan. They were serotyped by the indirect haemagglutination test according to the Standard Operation Procedures (SOP). Reference antisera of *Pasteurella multocida* and of *Mannheimia haemolytica* types were obtained from the OVI. *Pasteurella multocida* types A (ATCC=12945) and D (ATCC=12948), and *M. haemolytica* type A (ATCC= 4270s) were used as controls. The results of serotyping for *Pasteurella multocida* were A_2:3, A_1:3, and A_1:3 and for *Mannheimia haemolytica* were A1, A2 and A2

Introduction

Mannheimia haemolytica (*M. haemolytica*) is a weak haemolytic, Gram negative coccobacillus, oxidase and catalase positive and non-motile. The organism is a common commensal on mucous membranes of most domestic animals and of worldwide distribution (Paulsen *et al*, 2006). Most animals are asymptomatic carriers of the organism despite its causation of several economically important diseases in sheep and other domestic animals. Pneumonic pasteurellosis in sheep is the most important disease induced by this organism. It is also associated with septicaemic conditions in domestic sheep and was isolated from domestic goat (Midwinter *et al*, 1985). It is believed; however, that pneumonic pasteurellosis is a secondary complication of primary viral respiratory system infection (Cutlip *et al*, 1993). Pneumonia caused by *M. haemolytica* or *Pasteurella multocida (P. multocida*) may occur in all age groups of sheep, but it causes most economically significant losses in lambs. Outbreaks of pneumonia are usually not simple, especially in groups of lambs. *P. multocida* is an opportunistic pathogen in domestic and wild animals as well as in man. The bacterium was associated with various enzootic pneumonias in cattle, sheep and goats. It is also associated with certain forms of mastitis in cattle and sheep and it is found in upper respiratory tract mucous membrane of apparently healthy individuals (Barbour *et al*, 1997).

The most important disease induced by *P. multocida* in ruminants is haemorrhagic septicaemia (HS) which occurs most commonly in cattle and water buffalo in South Asia and Africa resulting in many mortalities.

Various serological procedures such as slide agglutination, tube agglutination, and ELISA have been used to quantify humoural antibodies against *P. multocida* and *M. haemolytica*. A

serotyping system using an indirect haemagglutination (IHA) test was developed to study the relationship between serotypes and biotypes of *P.* (now *Mannheimia*) *haemolytica* (Biberstein *et al*, 1960). This method was also previously used for serotyping *P. multocida* (Carter, 1955). Biberstein and co-workers (1960) reported a consistent association between serotypes and biotypes and distinguished 11 capsular serotypes. The number increased gradually to 17 serotypes (Fodor *et al,* 1984; Younan and Fodor, 1995; Angen *et al,* 1999). The IHA test was used in the present study to serotype isolates of *M. haemolytica* and *P. multocida* associated with sheep pneumonia in El-Damazin district, Blue Nile State, The Sudan.

Materials and Methods

Antigen preparation for *M. haemolytica* and *P. multocida*

Six field isolates of *M. haemolytica* and *P. multocida* (3 each) and the controls of *P. multocida* strains types A (ATCC12945) and D (ATCC12948) and *M. haemolytica* strain type A (ATCC4370s) were used for antigen preparation. Pure cultures of the isolates and the control strains were streaked onto two Tryptose Blood Agar plates (TBA) and incubated overnight at 37˚C aerobically. Cultures of each bacterium were scraped off the two TBA plates into a centrifuge tube containing 5 ml of phosphate buffer saline (PBS) with a pH adjusted to 6.0. The tubes were sealed with cloth plugs and placed in a water bath at 60˚C for 30 min. For *P. multocida,* 0.2 ml of hyalurinidase was added to each tube and the suspensions were incubated at 37˚C for 4 h. with frequent shaking every hour. The suspensions of *P. multocida*, *M. haemolytica* and the controls were centrifuged at 2000 rpm for one h. The supernatants were each collected with Pasteur pipettes and placed in a clean McCartney bottle and stored at -20 °C until used.

Preparation of guinea pig red blood cells

10 ml aliquots of heparinized guinea pig blood samples were obtained from Onderstepoort Biological Products (OBP). The blood was well mixed by inverting the tubes 10 times, and then it was divided equally into two centrifuge tubes and centrifuged at 3000 rpm for 10 min. The plasma was removed from each tube with a Pasteur pipette, normal saline was added to each deposit and mixed well by inverting the tubes gently 10 times and centrifuged at 3000 rpm for 10 min. The above steps were repeated twice and the packed RBCs were stored at 4 ºC and used in the next day.

Typing procedures

Standard Operation Procedures (Michel and Gelaw, 2007) were used for serotyping the isolates. One ml of healthy rabbit serum was added to each of 200 ml of normal saline in five sterile 250 ml measuring beakers. The extracts of *P. multocida, M. haemolytica* and the controls were removed from the freezer, placed in a water bath at 37 °C and left until defrosted. 0.2 ml of the washed guinea pig RBCs were added to each extract, and incubated at 37˚C for 90 min. Sensitized RBCs were each delivered into a beaker containing normal saline. With a multichannel pipette (Funnipipette, Finland), 50 µl normal saline were placed each in the 96 wells of U-shaped microtitre plates. Subsequently, 50 µl amount of reference antisera of *P. multocida* types A_1:3 , A_2:3, B_1:2, B_3:2, B_1:6 ,B_3:6, D_1:I, D_3:II and E:12 and of *M. haemolytica* types A2, T3, T4, A5, A6,T10, A12 ,A13, A14, T15 and A16 were added to the first row of each plate. The antisera were diluted in a twofold dilution from column A to H, and 50 µl volumes were discarded from rows of column H. 50 µl amount of the isolates and the controls extracts sensitized with guinea pig RBCs were added to each well of the five plates and of *M. haemolytica* to each of the 10 plates. The plates were left on bench at room temp for two hours and read for agglutination.

Results

Positive agglutination was confirmed by the presence of a dense clot at the bottom of the microtitre well and negative result by the presence of uniform suspension of the RBCs. The three isolates of *M. haemolytica* were found to belong to serotypes A1 (one isolates) and A 2 (two isolates) and of *P. multocida* were A_1:3 (two isolates) and A_2:3 (one isolate).

Discussion

M. haemolytica and *P. multocida* are important pathogens of ruminants in the Sudan, and knowledge of the existing serotypes is a prerequisite for developing preventive measures against pneumonic pasteurellosis in ruminants. The primary diseases induced by *M. haemolytica* are pneumonia in sheep and calves and septicaemia in lambs (Barbour *et al,* 1997). *P. multocida* serotypes B:2 and E: 2 are associated with haemorrhagic septicaemia in cattle and buffalos while serotype A is associated with pneumonic involvements in sheep and cattle (Harper *et al,* 2006). The indirect haemagglutination test (IHA) was used to study the relationship between serotypes and biotypes of *P.* (now *Mannheimia*) *haemolytica* (Biberstein *et al*, 1960) and they distinguished 11 capsular serotypes. The number of serotypes increased gradually to 17 (Fodor *et al*, 1984; Younan and Fodor, 1995). Serotypes of *M. haemolytica* strains in this study are A1 and A2 and of *P. multocida* A_1:3 and A_2:3. These findings are in agreement with those of other investigators; Shigidi (1976) found that 120 (30%) of the isolates, from nasopharynx of healthy sheep, were *P.* (*M.*) *haemolytica.* The report showed that 65% of *M.* (*P.*) *haemolytica* were biotype A strains and only 2.5% were biotype T. The latter study has suggested that apparently healthy adult sheep in the Sudan appear to have a relatively high incidence of carriage of *M. haemolytica*. El-Sanousi *et al* (1978) isolated *P. multocida* and *Actinobacillus ligneiresii* from dead and sick sheep in El Hoda farm. Fadia (1998) studied bacteria associated with sheep pneumonia, and isolated *M. haemolytica*, but did not type the isolates to strains level. Ilham and Keles (2007) found that 86.3% of their 66 isolates of *M. haemolytica* were of the serotype A. No cross reactions were observed among different antisera; this finding is in agreement with the findings of Chengappa *et al* (1984) in that no cross reactions occurred when strains of *M. haemolytica* were serotyped by the IHA test, but cross reaction did exist when they were serotyped by the rapid plate agglutination and counter-immunoelectrophoresis. This indicates that the IHA test is more specific than rapid plate agglutination and counter-immunoelectrophoresis tests. Large scale surveys should be conducted to isolate the strains of *M. haemolytica* and *P. multocida* associated with pneumonia in sheep and to identify them to serotype level.

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