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BACTERIOLOGICAL AND SEROLOGICAL STUDIES ON PASTEURELLA MULTOCIDA INFECTION IN RABBITS

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دراسات بكتريولوجية وسيرولوجية على الباستريلآ ملتوسيدا في الأرانب

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الباستريلا ملتو سيد ا ميكروب مخاطي يستعمر الجزء العلوي من الجهاز التنفسي للأرانب. الميكروب يحدث العدوى التنفسية ولكن يستطيع أن يخترق الأنسجة ويصل الجهاز الدورى محدثا خراريج أو حالات مرضية. الباستريلا ملتو سيدا تنتج مادة تسمى سياليديز وهذه المادة تحتوى على إنزيمات يعتقد أنها تساعد أو تمكن الميكروب على نمو واستعمار القناة التنفسية للأرانب واحداث المرض. استخدام الاليزا مبنى على أن الإنزيمات الموجودة في السياليديز للباستريلا مالتوسيدا تنتج أجسام مناعيه في السيرم مع أنه لا يوجد أعراض مرضية ملحوظة على هذه الأرانب بالأضافه الى أن الأرانب المصابه اكلينيكيا يوجد بها أجسام مناعيه للسياليديز. استخدام الاليزا للكشف عن الأجسام المناعيه للسياليديز ممكن يكون مفيد للكشف عن هذا الميكروب فى الأرانب المريضه اكلينيكيا وأيضا عمل مسح شامل لبيان الأرانب الحامله للمرض.

SUMMARY

Pastruella multocida is a mucosal pathogen that colonizes the upper respiratory tract of rabbits. Respiratory infections can result, but also the bacteria can invade the circulatory system producing abscesses or septicemia. Pasteurella mulocida (P.multocida) produce extracelluler sialidase that encode enzymes which is believed to augment colonization of the respiratory tract and the production of lesions in the active infection. The recombinant antigen enzyme-linked immunosorbent assay (ELISA) based on the sialidas enzyme of P.multocida and rabbits that were experimentally colonized with P.multocida produce detectable immunoglobulin (IgM) and (IgG) in serum, although they demonstrated no clinical signs of pasteurellosis. In addition clinically ill pet rabbits

infected with P.multocida possessed IgM and/orIgG antibody against sialides. The sialides ELISA may be useful for the diagnosis of P.mulocida infection in sick rabbits as well as for screening for carriers rabbits.

Key words: *Rabbits, Pasteurella multocida, ELISA.*

INTRODUCTION

Pasteurella multocida can be a virulent pathogen of rabbits producing fatal septicemia,pneumonia,chronic rhinitis and otitis media as well as multiple abscesses, however some rabbits are persistently colonized and exhibit no apparent signs of disease (Lu, *et al.*, 1978). Many rabbits become colonized with

Pasteurella soon after birth and after weaning more than 75% of rabbits that nurse from infected dams become culture positive (Holmes, *et al.*, 1984). The prevalence of P.multocida in clinically healthy rabbits ranges from 20-90%, Depending on the methods used for detection, as well as the age health status of rabbits (Glass and Beasley, 1989 and Lu, *et al.*, 1978). Laboratory rabbits colonized with P.multocida often develop clinical disease after being shipped to a research facility, but persistently colonized asymptomatic rabbits have been shown to produce aberrant results if they are used in research (Richard, *et al.*, 1997). The effect of pasteurellosis on biomedical research is so profound that continuous screening of research rabbits colonies is recommended (Ward, G.W. 1973). Culture of nasal swab specimens has been shown to be unreliable for screening, since up to 30% of infected rabbits may not be detected by this method (Holmes, *et al.*, 1986 and Holmes, *et al.*, 1987). In addition, serological screening has not been effective in identifying all persistently colonized rabbits because most of the serological tests use uncharacterized antigen mixtures that may not detect the multitude of serotypes that colonize rabbits (Delong, *et al.*, 1992; Kawamoto, *et al.*, 1994; Klassen, *et al.*, 1985; Lukas, *et al.*, 1987. Manning, P.J. 1984 and Zaoutis, *et al.*, 1991) Vaccination is not commercially available because of lack of efficacy, and furthermore, antibiotics may be effective for resolving the symptoms in sick rabbits but usually do not clear the bacteria from colonized rabbits (Gaertner, D.J. 1991; Mahler, *et al.*, 1995 and Welch, *et al.*, 1987). P.multocida isolates vary in their abilities to produce disease in rabbits, some are associated primarily with upper respiratory disease, while others cause septicemia, abscesses and pneumonia (DiGiacomo, *et al.*, 1991).

However, in order to initate infection, the bacteria must colonize the respiratory mucosa, and organisms that inhabit mucosal surfaces frequently produce sialidases (Corfield, T. 1992 and Vimer and Lichtensteiger, 2002). Sialidase is the only extra-cellular glycolytic enzyme probably plays a major role in the ability of P.multocida to colonize animals (Drazeniek, *et. al.,* 1972; Lee, *et al.,* 1988 and Scharmann, *et al.,* 1970), suggesting that this antigen may be useful for the serological diagnosis of pasteurellosis. In this study we report on the use of enzyme-linked immunosorbent assay (ELISA) to detect P.multocida infection in healthy and clinically ill rabbit.

MATERIALS and METHODS

Bacterial field isolate: 40swabs of lesions or nasal exudates with serum samples were acquired from rabbits from different localities at sharkia governorate with clinical signs suggestive of pasteurellosis. These swabs were streaked onto blood and chocolate agar plates for P.multocida (Knight, *et al.,* 1983 and Avirl, *et al.,* 1990)

Reference strain: P.multocida reference strain were obtained from veterinary serum and vaccine research institute, abbasia, Cairo.

Experimental animal: 13 male New Zealand rabbits 4-6week of age were used for production of antibodies and challenge.

Production of antibodies: Antibodies against P.multocida was produced by intradermal injection of 200ug of purified antigen in 500ul of complete freund adjuvant in 20 different sites on one New Zealand white male rabbit. The rabbit was vaccinated twice subcutaneously with 100ug of antigen in Freunds incomplete adjuvant at 3 weeks intervals. Preimmune blood samples were collected before the 1st immunization and serum was collected 14 day after each vaccination (Brogden, *et al.,* 1983).

Rabbit challenge: 12male New Zealand white rabbits (4-6weeks of age) were obtained from Pasteurella free vendor. The rabbits were housed in stainless steel cages. 150 gm of commercial pelleted feed was provided daily and water was available ad libitum. Serum samples were collected from 12 rabbits, 2 weeks after they were housed, in order to establish a negative baseline for ELISA. The rabbits were divided into 3 groups (each of 4 rabbits), the first group were kept as control without any treatment, the second group were administered intranasally 10 ul of phosphate buffer saline containing approximately 10 CFU of reference strain, these rabbits become systemically ill within 30 hours post

exposure and were administered 10 mg of enrofloxacin/kg body weight subcutaneously twice daily for 5 days. Serum was collected from the rabbits prior to challenge and weekly thereafter for the next 5 weeks. and the third group with the field isolates, serum was collected from the rabbits prior to challenge and weekly thereafter for the next 11 weeks. On week 12, the rabbits were sacrificed and then sample swabs were taken from the deep nasal turbinates, pharynx, trachea, ear bullae and cervical lymph nodes for culture. A full necropsy was performed on all of the rabbits in order to detect gross indications of infections.

ELISA: a whole cell-lysate ELISA was performed as described by (kawamoto, *et al.,* 1990 & 1994 and Bonga, *et al.,* 1997) in order to confirm the infection status of rabbits.

RESULTS and DISCUSSION

The term pasteurellosis can be used to refer several condition caused by P.multocida that harbored in the nasal cavity of rabbits leading to rhinitis or asymptomatic chronic infection. The importance of pasteurellosis in rabbits is underscored by the fact that its clinical syndromes are refractory to antibiotic administration and there no reported vaccination strategy that can prevent all the manifestation of the disease (Manning, *et al.*, 1994).

Colonization and disease is influenced by factors related to both host and pathogen. Three strains of P.multocida have been isolated from rabbits. They are classified by capsular type and serotype into A:12, A:3, 2:D and B in which A:12 is the most common in rabbits but the more sever disease has been associated with A:3.

The reference serotype and field isolates of P.multocida tested produce sialidase activity in the filter paper spot test (Mizan. *et. al.,* 2000).

All isolates including the reference isolate suggesting that this antigen is widely distributed among the serotypes of P.multocida when they were used in whole-cell ELISA, suggesting that all serotypes tested produce an antigenically conserved the antigen (sialidase). These results suggest that sialdise may be useful antigen for the serological screening of rabbits infected with P.multocida.

Sialidase gene (antigen) has been shown to be difficult to purify from P.multocida, because it is membrane associated (Mizan, *et al.*, 2000). When this antigen protein was used to vaccinate a rabbits, high titer antibodies serum was acquired. The serum antibodies was used as

positive control serum in the ELISA. All rabbits used in this experiment were nasal swab culture and ELISA negative for P.multocida and demonstrated no symptoms of disease prior to testing by ELISA or challenge. Sera from these rabbits were tested for cross-reactive antibodies against this antigen by ELISA. Science cross-reactive antibodies were most apparent at the lower dilutions, we chose dilution of 1/16(IgG) and 1/18(IgM) as the minimal dilutions for the detection of antibody titers.

Serum from vaccinated rabbits, which was exsanguinated 2 weeks after the third immunization served as the positive control for the IgG ELISA, serum removed from these rabbits 2week after the first immunization served as the positive control serum for IgM ELISA and the sera from the negative rabbits were also used as the negative control for the antibodies (IgM) ELISA.

The immune system of colonized rabbits may have become exposed to P.multocida surface protein during colonization and bacterial cells may also have been phagocytosed by antigen processing cells present in the mucosal tissue. In order to determine whether antibody could be detected in colonized rabbits we challenged rabbits intranasally with several P.multocida isolates. 3rabbits (2^{nd} group) that are administered highly virulent (reference) isolate exhibited symptoms of septicemia on the day after challenge and three rabbits (3^{rd} group) that are administered the field isolates (cocktail) show symptoms of snuffles. The rabbits of 2^{nd} groups were treated with antibiotic that resolved the infection, and all of the rabbits recovered. Serum from the rabbits did not display antibodies, and P.multocida was not detected post-treatment by ELISA or culture. However two of the four rabbits from this group that were tested by whole cell-lysate ELISA produced positive titers 2-3 week post challenge, confirming their P.infection status. The rapid onset of disease, coupled with therapeutic treatment, probably resulted inadequate exposure to P.multocida surface sialidase and the antibiotics have been only partially successful in controlling infection, since they do not completely eliminate the bacterium (Gaertner, 1991 and Jaslow, *et al.*, 1981).

In contrast, the rabbits that were administrated a cocktail of the field isolates (3^{rd} group) produce IgM antibodies and one rabbit demonstrated IgG antibodies 6 weeks after inoculation. Since we used anti-whole-molecule for detection, the IgG titer was actually nonisotype specific IgG. The IgG levels continued to rise in these rabbits, but one rabbit exhibited a positive titer only after 11 weeks postexposture. IgM

titers were transient and undetectable in the samples collected at 9 weeks postexposture .P.multocida was detected in rabbits of these group 14 week post inoculation by ELISA and nasal swabs collected.

At necropsy P.multocida was also isolated by culture from tracheal swab specimen of rabbits that demonstrated the highest IgG levels post inoculation.

Neither rabbit exhibited any clinical signs of P.multocida infection, nor were lesion detected by gross pathology, suggesting that the rabbit that colonized but not adversely affected by the bacteria. While serum samples from these rabbits produced increasing values by Kawamoto ELISA over the course of the experiment, only one serum sample from one rabbit had a positive titer.

These results suggest that this antigen (sialidase) is sufficiently expressed by P.multocida during surface colonization of the respiratory mucosal system to stimulate serum antibody production. Sera and swabs collected from control group were negative for ELISA and cultures. These results suggest that the ELISA may be useful for identifying healthy carrier rabbits. Because of its virulence, early diagnosis of P.multocida infection in pet rabbits is critical. Exposure to virulent invasive strains may result in rapid penetration of the respiratory mucosa, leading to per acute or acute disease (Al-Hadawi, *et al.,* 1999; Al-Haddawi, *et al.,* 2001; Lee, *et al.,* 1994 and Rabier, *et al.,* 1997). In the challenge with reference strain described in this report, we observed rapid infection, in which the rabbits were clinically ill within 30 h. after intranasal exposure to a low bacterial dose. Similar results have been reported by others using other virulent P.multocida isolates in rabbits (Al-Haddawi, *et al.,* 2000; Al-Haddawi, *et al.,* 2001 and Al-Lebban, *et al.,* 1988). These infection might be detectable by PCR or culture because of the high replication rate of bacteria in the tissue (Lee, *et al.,* 1988). Although culture is considered the gold standard for detection ,techniques for the isolation and identification of P.multocida are time consuming and often fail because some transport media, including commonly used for transport swabs,do not maintain P.multocida viability for more than 1day at room temperature (Kawamoto, *et al.,* 1997). In some clinical cases, the organism can not be cultured from obviously diseased organs because the animals may have received antibiotics prior to sample submission. In sick animals that exhibit per acute disease, an ELISA may not be useful for the detection of antibodies post infection if the animals have been treated with antibiotics.

It is also important to determine healthy research rabbits are P.multocida free. The bacteria can be present at low numbers deep in the nasal turbinates of carrier rabbits, rendering their detection by culture impossible without killing the animal (Holmes, *et al.,* 1987 and Ward, G.M.1973). Several research groups have investigated ELISA as a method for the detection of colonization of healthy rabbits with P.multocida (Holmes, et al.1986; Hwang, *et al.,* 1986; Kawamoto, *et al.,* 1994; Klassen, *et al.,* 1985; Lukas, *et al.,* 1987; Peterson, *et al.,* 1997 and Zaoutis, *et al.,* 1991). However, rabbits exposed to low-virulence strains may become persistently colonized (DeLong, *et al.,* 1992), but the bacteria may be primarily associated with the mucus layer or may be adhere to the surface of mucosal epithelial cells (Al-Hddawi, *et al.,* 2000; Glorioso, *et al.,* 1982). The ELISA can detect this colonization state in chronically colonized animals. (West, *et al.,* 2002) demonstrated that serology with extra cellular proteins or cell lysates could detect colonization 6-12 months before culture detection, analogous to the screening of rabbits for P.multocida infection.

Stress or shipping can reduce rabbit mucosal defenses and allows the bacteria to invade the mucosal surface, with subsequent host exposure to other bacterial antigens. Low virulence strains would be more likely to produce chronic infections, which would result in high anti-P.multocida antibody titers in serum that could be detected by serological methods. Most serological tests use boiled whole P.multocida cells, heat-stable cell lysates, or purified lipopolysaccharides (LPS) as the antigen, which can result in both high background levels and significant numbers of false-positive or false-negative results (Cary, *et al.,* 1984; Hwang, *et al.,* 1986; Manning, P.G. 1984 and Manning, *et al.,* 1986). Infected rabbits may make high levels of LPS-specific antibody (Cary, *et al.,* 1984; Manning, P.G. 1984 and Manning, *et al.,* 1986) But the diversity of P.multocida serotype-specific LPS ensures that some strains may not be reactive by all assays (Brogden and Packer, 1979; Chengappa, *et al.,* 1982 and Rimler and Brogden, 1986)

A better target for P.multocida detection would be a homogeneous antigen that could be easily purified and that is present in all isolates. In order to obtain a more homogeneous antigen for serological testing, purified recombinant sialidase genes are used. It was shown that sialidase is associated with P.multocida outer membrane and dose not exhibit a high degree of homology to other sialidases that have been characterized (Mizan, *et al.,* 2000).

In the present study, we demonstrated that the antigen is ubiquitous in P.multocida isolates that cause disease in domestic animals. Many organisms that colonize the respiratory system produce sialidase, and this enzyme functions in the removal of sialic acid from mucus, which allows sialidase- producing bacteria such as P.multocida to access sialic acid as an energy source (Corfield, 1992; Mizan, *et al.*, 2000 and Vimer, and Lichtensteiger, 2002). We have shown that expression of sialidase during colonization or chronic infection of deep host tissue elicits humoral response, which enables detection of persistent colonization by serology.

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