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STUDY THE PATHOGENICITY OF *PASTEURELLA MULTOCIDA* IN MICE

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ABSTRACT

All Haemorrhagic septicaemia (HS) strains of *Pasteurella multocida*, fell into Roberts type I, *P. multocida* Roberts I: Carter B, the serotype is responsible for Asian HS. This study conducted to evaluate the validity of the mouse as model for HS in cattle, buffaloes, goats. A total of thirty six Swiss mice of both sexes divided into two main groups (infective and control) and each group subdivided into three groups (1^{st}, 2^{nd}, 3^{rd}), each group subdivided into 2 subgroups (A, B). Each infective subgroups infected with different route (I.P and S.C) and different dose of the (Robert I :carter group B) strain as 10^4 cfu, 10^2 cfu and 50 cfu respectively for both routes. Many criteria (clinical sings, mortality, bacterial isolation from different organs, and gross pathological changes) were used to describe infection in mice as a tool for further investigation in some large animals. The study showed that mice displayed fever, depression, anorexia, and other clinical signs 24hrs. before death which occurred between 24-72 hrs.

P. multocida: Robert I: carter : B was isolated from most organs of 1^{st} and 2^{nd} group infected with (10^4 and 10^2 cfu) but not isolated from the brain of third group infected with 50 cfu (I.P and S.C) also from kidney and lung of S.C B3 of the infected mice, and from all organs of the third mouse of this subgroup.

Mice showed different gross pathological changes of internal organs and brain , these characterized by splenomegaly, hepatomegaly, petechial haemorrhage similar to that observed in cattle, buffaloes.

These results suggest that the mouse would seem to provide an ideal tool to study HS in cattle, buffalo.

INTRODUCTION

P. multocida is recognized as an important veterinary pathogen ,causes diseases in a wide range of animal species and is the causative agent of numerous, economically important disease, including avian fowl cholera, haemorragic septicemia, enzootic pneumonia and swine atrophic rhinitis (1).

In Iraq, P. *multocida* was incriminated in many infections, Makkawi, *et al*. (2) were isolated *P. multocida* from sporadic cases of gazalles in a social sector field. Also *P. multocida* serotype A:1 was the causative agent of fowl cholera outbreaks occurred in parent stock layers of various ages and breeds in Muradia and Tharthar governmental poultry farms which occurred during the period between March and August, and it was recorded for the first time (3).

During an outbreak of Pasteurellosis in mountain goats, gazelles and deers in a social sector field, 7 (6.14%) strains of *P. multocida* and 55 (48,24%) of *Mannhaemia .haemolytica* were isolated (4). *P.multocida* was the causative agent of an outbreak of Pasteurellosis in buffalos in Thaqar province (unpuplished data). Also 40(29.4%) and 23(16.91%) isolates were isolated from field animals and humans respectively, and the isolation of *P. multocida* from human occurred for the first time in Iraq(5). Al-helaly (6) mentioned that P. multocida isolated from patients suffering from respiratory diseases.

Roberts (7) developed a system of *P. multocida* based on passive protection tests in mice, this was the first classification to meet some degree of acceptance ,he was able to identify four types I, II, III and IV. Since all HS strains fell into Roberts type 1, This designation become fairly well established and Subsequently, Hudson (8) added fifth serotype.

Mouse is known to be very sensitive to *P. multocida* infection, hence intra-peritoneal (I.P) inoculation of mice is the choice not only for isolation from mixed bacterial population (9), but also it could be used for the estimation and comparision of strain pathogenecity (10).

Also mouse has been used in passive protection tests with *P.multocida* for defining the protective antibody response of cattle and buffalo to vaccination (1,3,4; Ramdani unpubl. data). The mouse has also been used as a model for avian pasteurellosis, fowl cholera (11). Despite these uses, the validity of studying *P. multocida* and more specifically HS infections in mice has not been evaluated nor has the disease been critically compared with the disease in cattle in Iraq. Nevertheless, mice represent the ideal tool by study HS as they are immunologically and genetically defined, easy to manipulate and inexpensive to maintain. The validation of such a model require warrant further studies in order to be able to critically assess it as a model for this economically important disease (12).

The aim of this paper was to describe infections in mice with Robert

:I,Carter:B. an HS type strain of *P. multocida* and to critically assess the usefulness of mice as a tool to further understand the disease in cattle. The parameters of bacterial pathogenicity of three dose of bacteria through two routes of infection and gross pathology of disease were defined in this study as a basis for establishment of rigorous comparison between the disease in cattle, buffaloes and goats, and the infection parameters in mice.

Materials and Methods:

A- Bacterial strain &infective doses preparation:

P. multocida strain Robert- 1- supplied by Al-Kindi Company for Veterinary Drugs & vaccines production, Baghdad-Iraq. Some of biochemical tests were carried out to confirm diagnosis according to (13). Then the strain was cultured on Tryptic soya broth containing 0.6% yeast extract, incubated at 37c for 16hrs. Then the culture was washed with PBS (pH=7.2) three times & three dilutions were prepared as infective doses(10^4,10^2,50) cfu/ml.(14), depending upon the LD50 reported by (15) who used (10^2 cfu), for the same strain which was supplied by Alkindy company and used in the current study .

During three days following the infection, clinical signs and mortality rate were observed.

B- Experimental Design:

Thirty six healthy Swiss mice of both sexes aged between 7-8 weeks, weighted 13-16g were divided into two main groups (infective group consist of 18 mice, control group consist of 18 mice), infective group was divided into three groups, each group subdivided into two subgroups, the subgroup of 1st group (A1,B1) infected with 10^4 cfu.(0.1ml) (I.P) and subcutaneously (S.C) (n=3 per route). The subgroup of 2nd group (A2,B2) ,and 3rd group (A3,B3) were infected with 10^2 cfu, 50 cfu respectively with the same route and dose as above. Control groups were injected with 0.1ml PBS by the same routes as infected groups. Then the clinical signs, mortality, bacterial isolation from internal organs (liver, spleen, lung, heart, kidney and brain) and gross pathological changes for the infected and control mice were recorded, these included location, color, size, shape, consistency and appearance of cut surface.

Results:

Clinical signs:

Clinical signs which were observed on mice post infection during the first 24hrs, and before death, were included fever, depression, anorexia, dullness, ataxia and cyanosis of extremities and crusting around the eyes and nose. The result showed mortality of mice between 24-72 hrs. after infection. The mortality rate of mice after 24hrs, means the lower survival time mean which equal to 24hrs in the subgroup A2(I.P), A3(I.P) and

B3(S.C) with infective dose : 10^2, 50, 50 cfu respectively, while the mortality rate after 72 hrs, means the highest survival time mean which equal to 56 hrs, appeared in subgroup B2 (S.C) infected with 10^2 cfu (table: 1). Clinical signs and morbidity began to occur approximately 6 hrs earlier in mice infected with 10^4 , 10^2 than mice infected with 50 cfu.

Table (1): Survival of mice infected with *P.multocida* with doses and routes of infection

**Survival time mean	Survival time No. of died mice/time*			Dose/rout of infection	subgroup	group
	72	48	24			
43	-	2	1	1×10^4cfu /ml I.p	A1	1st
32	-	1	2	1×10^4cfu /ml S.C	B1	
24	-	-	3	1×10^2 cfu /ml I.P	A2	2nd
56	2	-	1	1×10^2 cfu /ml S.C	B2	
24	-	-	3	5×10 cfu /ml I.P	A3	3rd
24	-	-	3	5×10 cfu /ml S.C	B3	

*each subgroup consists of 3 mice.

**survival time means in hour.

Bacterial isolation:

The results showed that *P. multocida* was isolated from all organs of mice which infected I.P and S.C with (10^4,10^2) ,while it was not isolated from the brain of mice that infected with dose 50cfu (I.P and S.C) and from kidney and lung of (S.C B3) infected mice, also from any organ of the third animal of the same subgroup. (table :2).

Table 2: Bacterial isolation from internal organs of the infected mice.

organs						No. of mice	Dose/route	subgroup	group
Brain	**Kidney**	**Lung**	**Heart**	**spleen**	**Liver**				
+	+	+	+	+	+	1	1×10^4cfu I.p	A1	1st
+	+	+	+	+	+	2			
+	+	+	+	+	+	3			
+	+	+	+	+	+	1	1×10^4cfu S.C	B1	
+	+	+	+	+	+	2			
+	+	+	+	+	+	3			

+	+	+	+	+	+	1	1×10^2 cfu I.P	A2	2nd
+	+	+	+	+	+	2			
+	+	+	+	+	+	3			
+	+	+	+	+	+	1	1×10^2 cfu S.C	B2	
+	+	+	+	+	+	2			
+	+	+	+	+	+	3			
-	+	+	+	+	+	1	5×10 cfu I.P	A3	3rd
-	+	+	+	+	+	2			
-	+	+	+	+	+	3			
-	-	-	-	-	-	1	5×10 cfu S.C	B3	
-	-	-	+	+	+	2			
-	-	-	+	+	+	3			

Gross pathological changes:

The internal organs liver, spleen, kidney, heart, lung and brain and the site of injection were examined to detect the gross pathological changes due to *P.multocida*.

Mice showed obvious gross lesions differ in severity from one animal to other and from one group to other, liver showed slight enlargement, congestion, petechial hemorrhages and friable in cut surface, in addition to yellowish or yellowish white necrotic foci. The spleen showed moderate congestion, splenomegaly and multivariable size, yellowish foci appeared on their surface and parenchyma in mice died within 48-72 hours , the kidneys showed cortical congestion and they were soft through cut section

The lungs showed moderate to severe congestion and petechial hemorrhage, solid in cut section and gray raised consolidated areas. The cut surface showed foamy edematous consistency, also there was pleural adhesion in some areas. The heart appeared slightly rounded and solid in cut sections. While the lung, heart, spleen, of two mice of sub group B3 (S.C) appeared normal.

The brain showed mild to severe congestion, also the site of injection showed sever congestion and petechial hemorrhage in all animals, while the kidney and spleen were normal in subgroup A3 (I.P), also the lung, heart and spleen of two mice of subgroup B3 (S/C) appeared normal.

DISCUSSION

The results of this study describe *P. multocida* infection in mice using the haemorrhagic septicaemia type strain (Robert 1, carter B). All the parameters examined suggest a role for the mouse as a tool to study HS and are sufficiently encouraging to

warrant further rigorous studies to be undertaken to evaluate the validity of the mouse as a model for HS in cattle, buffalo. Also mice are exquisitely susceptible to *P. multocida* with our study showing that 50 viable organisms constituted a critical pathogenic dose, this acceptant with (12) they concluded that as few as 20cfu produced an overwhelming septicaemia in mice. Also Heddleston et al, (16) mentioned similar observation using M1404 strain in Swiss Webstor mice. Also Bain (17) recorded that as 2 organisms produce 100% mortality in mice.

Clinical signs began to occur approximately 6 hrs. earlier in mice infected with 10^2,10^4 CFU, than mice infected with 50 CFU and result in mortality within 24-72hr. of infection, this indicated the lower survival time mean, (24hrs) appeared in the subgroups A2(I.P) and A3 (I.P) regardless the infective doses, this result agreed with (12) when injecting 20 CFU (I.P) in mice produced an overwhelming septicaemia in mice in less than 30 hrs. The kinetics of infection demonstrated a very rapid *in vivo* multiplication rate, there was no evidence of inhibition of bacterial cell growth by natural host defense mechanisms (peritoneal macrophages), even with the very small inoculum used (13).

The results of the S.C groups that the clinical disease occurred within 24 -72hrs after infection with the same doses injected S.C this indicated that subgroups B3(S.C) infected with 50 cfu showed the lower survival time mean (24hr) , these results were supported by previous studies which explained that endotoxaemia of gram negative organisms can initiate septic shock due to the proliferation of microorganism at the site of infection and release endotoxin and exotoxin which stimulate endogenous mediators of sepsis which lead to death(18), while subgroup B2(S.C) showed highest survival time mean (56hrs) , although they were infected with different dose 10^2 through the same route , this results may be discussed by the potential ability of microorganism to evade the host defense mechanism and survive within phagosome so they act as a host protein which was not recognized early by both humoral and cellular immune response.(19,13), this is also depending upon idea that S.C route of infection was considered the best method of experimental recreation of the disease in goats, as those of cattle since 3 (60%) goats needed to be killed per acutely in experimental infection of *P. multocida* in goats (20). Similar observations were made in cattle and buffaloes revealed that subcutaneous inoculation resulted in death within 24-31 and 60hr. for buffaloes and cattle, respectively compared to goat that took 48hr (21). The difference in susceptibility of mice in different groups could be attributed to an interaction between a number of factors such as burden of infections, individual defense mechanism, and immunity level (1) .

Clinical signs which were observed on mice post infection were in agreement with (22) who mentioned that the HS is a peracute septicaemia, the portal of entry is ill-defined, with death occurring 12-48 h after the onset of symptoms of HS which include high fever, shaking, depression and reluctance to move also there is a nasal discharge and excessive salivation . These signs and the rapid nature of the disease are similar in mice and cattle, and mice also demonstrated naso-ocular involvement with a degree of 'crusting' around the nose and eyes.

The crusting was found to be due partly to the excretion of *P. multocida* organisms

in the murine tear glands which resulted in the high bacterial counts seen in the peripheral blood samples during the early time points of the kinetic experiments (12). Also in experimental study conducted by Al-Humam et al. (23) showed that calves experimentally infected with *P. multocida* type A demonstrated typical clinical signs of haemorrhagic septicaemia ; which were similar to signs observed on the infected mice.

Ataxia is considered as a neurological signs which was observed, caused by brain lesions, this in agreement with Savage and Sheldon (24) who reported similar results after I.P infection of mice with *P. multocida.*

Death of animals in all infected groups indicated that the mice were exposed to infective dose of highly virulent *P.multocida* which overcome innate immunity and rapidly replicated and disseminated from the site of inoculation to the internal organs that cause bacteremia, septicemia, septic shock and death of infected mice (25).

These observations support the results of previous studies which explained that endotoxemia of gram-negative organism can initiate septic shock, the process began with proliferation of microorganism at the site of infection, and invade the blood stream directly or may proliferated locally and release various substances in the blood stream, these substances include endotoxin and exotoxin, which, in turn, stimulate the release from plasma precursors or cells (monocyte, macrophages, endothelial cells, neutrophils and others) of endogenous mediators of sepsis which lead to failure of multiple organs system, disseminate intravascular coagulation and death (18).

The results of bacterial isolation have revealed isolation of *P. multocida* from internal organs and brain, these results in agreement with results reported by (6), that *P. multocida* was isolated from internal organs (liver, spleen, heart, lung and kidney) of the control group in experimental infection in mice by *P. multocida* isolated from human and sheep.

Also the results were in agreement with Zamri- Saad and Shafarin (26) reported that *P. multocida* B:2 was successfully isolated from lungs, lymph nodes, spleen , tonsils, heart blood, liver and subcutaneous fluids of goats which were infected subcutaneously and killed peracutly in experimental infection in goats through 3 routes. Also in experimental study conducted by Al-Humam, et al., (23) showed that different organs were demonstrated to be predilection sites for *P. multocida*, these include lung, heart, trachea, spleen, liver, pharynx and oedematous fluid in the neck and nasal cavity.

Isolation of *P. multocida* from the brain is in agreement with (27), recorded that *P. multocida* was isolated from the brain of rabbits showing common clinical signs of head tilt. Also Rasheed (15) mentioned that *P. multocida* was isolated from liver, kidney, spleen and lymph nodes of rabbits experimentally infected with this pathogen.

Some organs of the third group appeared normal and P.multocida was not isolated from the brain of third group and from the kidney and lung of S/C infected mice also from any organs of one animal of B3 subgroup, these results may be attributed to individual defense mechanism and immunity level(1).

The gross pathological changes which were evident on examined organs, the petechial haemorrages and congestion of the liver and lung, these results were in agreement with Ramdani, et al (12), who mentioned that gross pathology of pasturellosis in mice was characterized by splenomegaly, lymphadenopathy and petechial haemorrhage similar to that observed in cattle and buffalo with HS.

Liver showed slight enlargement, congestion and petechial haemorrhage which changed to yellowish white foci, also the liver was friable these indicate fatty changes (28). Congestion of spleen and moderate splenomegaly with rounded edges related to hyperplasia of the organ (29,30).

Lung lesions were recorded in the results were congestion, petechial haemorrhage and consolidated areas and pleural adhesion and foamy contents, due to intra-alveolar edema (28, 31).

Kidney lesion was observed related to bacterial endotoxin (endotoxemia) (32). Petechial haemorrhage at the injection site is in agreement with Zamri-Saad and Shafarin (26), recorded that typical lesions of HS with petechial haemorrhage at the injection site, sever pulmonary congestion, odema and hydro thorax.

Methods of inoculation used in this study, I.P and S.C route, were effective in organs of infected mice because different pathological changes appeared due to *P. multocida*,which have established a primary site of infection and they multiply before spread to other organs. Infection can spread through the periton and via lymphatic system to the blood stream and incase of bacteremia, the bacteria spread widely in the body and permits them to reach tissue particularly suitable to their multiplication(33,15)

Considering these results it can be concluded that the mouse would seen to provide an ideal tool to study HS but warrant further studies in order to be able to critically asses it as a model for these economically important disease, is needed.

دراسة أمراضية جرثومة الباستوريلا ملتوسيدا في الفئران

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الخلاصة

تقع كل عتر مرض عفونة الدم النزفية تحت النوع (Roberts type I) ، ويعد النمط المصلي (Roberts I : Carter B) مسوولا عن مرض عفونة الدم النزفية في قارة آسيا . صممت هذه الدراسة لتقييم صلاحية اعتبار الفئران كنموذج للاصابة بمرض عفونة الدم النزفية في الابقار والجاموس. تم أخذ ٣٦ فارا سويسريا وقسمت إلى مجموعتين هما مجموعة الإصابة ومجموعة السيطرة ومن ثم قسمت كل مجموعة إلى ثلاثة مجاميع الأولى والثانية والثالثة ومن ثم قسمت تلك المجامـيع إلى ما تحت المجموعة (A and B) .حقنت كل مجاميع الإصابة بجرع وطرق إصابة مختلفة بجرثومة الباستوريلا ملتوسيدا (Robert 1:Carter B) فكانت الجرع ($50,10^2,10^4$ cfu) على التوالي لكلتا طريقتي الإصابة (تحت الجلد وفي الخلب) .

استخدمت عدة معايير وهي الأعراض السريرية والهلاكات والعزل البكتيري من عدة أعضاء والتغييرات المرضية العيانية لوصف الإصابة في الفئران للتعرف على المرض في بعض الحيوانات الكبيرة.

أظهرت الدراسة إن الفئران أصيبت بالحمى والخمول وفقدان الشهية واعراض سريرية اخرى خلال الـ٢٤ ساعة قبل الهلاك و هلكت الحيوانات بين 24-72 ساعة.

عزلت الجرثومة من كافة اعضاء الفئران للمجموعتين الأولى والثانية المحقونة بالجرعة ١٠^{٤} و ١٠^{٢} ولم تعزل من الدماغ لفئران المجموعة الثالثة المحقونة بجرعة ٥٠ (cfu) في داخل الخلب وتحت الجلد ولم تعزل من الكلية والرئة ، كما انها لم تعزل من جميع أعضاء الفأر الثالث من المجموعة المحقونة تحت الجلد .

أظهرت الفئران المحقونة عدة تغييرات مرضية عيانية على اعضاء الجسم الداخلية وكذلك الدماغ واتصفت تلك التغييرات بتضخم الطحال والكبد والنزف النقطي وهذا يشابه مايلاحظ في الابقار والجاموس .

أظهرت النتائج إمكانية اعتبار الفئران نموذجا لدراسة الاصابة بمرض عفونة الدم النزفية في الأبقار والجاموس.

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