Some bacteriological studies on toxiginic strains *of Pasteurella multocida* in calves

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Abstract
Haemorrhagic septicemia (HS) is an acute highly fatal, septicemic disease of cattle and buffalo and is one of the most economically important livestock diseases. It is caused by certain serotypes of *Pasteurella multocida*. A total of (284) samples were collected from calves, 200 nasal samples (123 from clinically affected and 77 from apparently healthy) and 84 from internal organs (lung, Liver, Trachea, lymph node, peritoneal and pericardial fluid) of dead calves. Gross lesions of animals died with respiratory infection were observed, and discussed. *Pasteurella multocida* was isolated with a percentage of 78.04% and 32.46 % from nasal swabs of diseased calves and apparently healthy ones respectively and 82.14% from the internal organs of dead calves. The pathogenicity of *Pasteurella multocida* was studied by recording the lethal effect in rabbit and mice, where all the rabbits died within 12hrs after the injection of the organisms, and in mice within 30hrs, and the gross lesions were recorded and discussed. The toxin of *Pasteurella multocida* was prepared by cell sonnication, and its toxigenic effect was studied through its lethal effect in mice and dermonecrotic activity in ginea pig. (The diameter of dermonecrotic lesions ranged from (4-5mm) at toxin titer 64 and 32 respectively. Formalin treated toxin was used to evaluate its potency for protection in mice and ginea pig. There is no lethal effect in immunized mice (100% survived), the necrotic activity was not observed on ginea pig immunized with the formalized toxin (100%).

Key words: *Haemorrhagic septicemia, Pasteurella multocida, dermonecrotic lesions, formalized toxin.*

Introduction

Haemorrhagic septicemia (HS) is an acute highly fatal, septicemic disease of cattle and buffalo and is one of the most economically important livestock diseases. It is caused by certain serotypes of *Pasteurella multocida* **(Bain *et al*., 1982)**. Buffaloes are more susceptible to HS than cattle **(De Alwis, 1999** and **Tabatabaei *et al*.2007)**. So far, various serotypes (A, B, C, D and E) of *P. multocida* have been detected among the livestock population in Asia and Africa **(Benkirane and De Alwis, 2002, Borkowska-Opacka and Kedrack, 2003 and Kumar *et al*., 2004).** The main habitat of *Pasteurella multocida* is respiratory tract. It is occasionally present in healthy domestic and wild ruminants **(Buxton and Fraser, 1977)**.

Calves play an important role in the development of the dairy sector, as the future of the dairy herd depends upon the successful raising of young calves. Female calves are especially kept for herd replacement. The male calves are usually kept up to weaning **(Ahmad *et al*., 2009).**

In cattle, capsular types A and D are associated with bovine enzootic pneumonia, and type A is the main type associated with bovine pneumonia **(Anonymous 2004; Christensen *et al*. 2004; Catry *et al*. 2005)**. It has been isolated from nasopharynx of healthy population **(Carter and De Alwis, 1989; De Alwis, 1992)**. It is thought that stress may play a role in causing the shift to an active carrier state. Outbreaks also tend to be associated with rainy season which is thought to be related to increased survival of the organism in wet conditions and shedding of the organisms by carrier animals occurs due to weather induced stress **(De Alwis *et al*., 1993)**,

Toxin production is another factor which influences virulence; toxin produced by bacteria

can cause disease by itself and in sites removed from where the bacteria reside. This has been shown with purified toxin while Hemorrhagic septicemia has been recognized for many years, The Mice would seem to provide an ideal tool for studding Hemorrhagic septicemia, but warrant further studies in order to be able to critically assess it as a model for this economically important disease **(Ramdani *et al.*, 1990** and **Chrisp and Foged, 1991)**.

The aim of this study was isolation and identification of *P. multocida* from calves determines pathogenicity in mice, preparation of toxin and studies its effect on laboratory animals, and to critically assess the usefulness of mice as a tool for further studies the disease in cattle.

Material and methods

A total of ((200) samples were collected by sterilized cotton swabs from calves aged (6-9month) out of them 77 nasal swabs from apparently normal healthy, and in contact with 123 calves with respiratory disorders in the form of nasal discharges and congestion, On the other hand 84 samples of internal organs (lung, Liver, Trachea, lymph node, peritoneal and pericardial fluid) were collected from dead calves.

Samples were packed separately in sterile plastic bags, labeled and transferred to the laboratory in ice box for bacteriological, biochemical and serological examination.

Isolation and identification of *P.multocida*:

The samples were cultured onto blood agar and MacConkey agar; cultured plates were incubated at 37°C for 24 to 48 hrs. After 48hrsthe isolated organisms were identified on the basis of cultural, morphological and biochemical characteristics **(Cruikshank, 1975)**. Colonies of the positive samples were grayish, translucent with entire edges. All strains were characterized with identification kits API 20NE. Gram staining, oxidase, catalase, and indole production, motility, growth in MacConkey agar, and fermentation of mannitol were evaluated. Isolates that were gram-negative rods, catalase, oxidase, and indole positive, fermented mannitol, were non-motile, and did not grow in MacConkey were considered to be *Pasteurella multocida* .All strains fermented sugars like glucose, sucrose and maltose while lactose was not fermented. Furthermore, the isolates were negative for Vogues Proskeurs, methyl red and gelatin liquefaction tests and did not hydrolyze urea.

Impression smears from the internal organs, nasal swabs as well as blood smears were prepared on microscope slides and stained with Gram's and Gemsa stains for direct microscopic study.

Congo red binding of *P. multocida*

It was done according to **Berkhoff and Vinal (1986)** with some modifications. All *Pasteurella multocida* isolates were streaked on Congo red media (BHI broth 27 g, agar 20 g, Congo red 0.03 g and distilled water 1000 ml), sterilized in autoclave at 15 lb for 20 min, then incubated at 37°C for 24 hours and kept at room temperature for another 24 hours and observed for appearance of orange colored colonies which were Congo red positive.

Pathogenicity

The pathogenicity of the isolates was studied in rabbits and mice as follows:

In rabbit:

Three rabbits were injected intraperitonially (I/P) with 0.2ml cultured isolates which incubated on peptone water at 37°C for 24hrs. One rabbit remained uninfected as control one.

In mice:

It was done by injecting five mice with 0.2ml of a log phase (3-6hrs of incubation) of isolates and left 3 mice as negative control.

Preparation of *P.multocida* toxin by cell sonication: (Nakai *et al*., 1984)

Each strain was inoculated on blood agar plate and incubated for 24hrs at 37ºC. The culture was harvested directly from the agar plates with normal saline solution 2ml/plate. The cells were sonnicated 3 times for 1 min each in cold water bath, then the sonicate was centrifuged at 10000 rpm for 30 min at 4ºC, then the supernatant was filtered through a sterile

0.22mm pore membrane filter. The toxin (supernatant) was kept frozen at -20ºC until use.

Estimation of toxin content:

The protein concentration in the OMP-rich extracts was estimated by the method of **Lowry *et al.* (1951)** using bovine serum albumin as standard.

Detection of *P.multocida* toxicity in culture supernatant:

The toxicity assay of *P.multocida* was evaluated by two methods:

a-The lethal toxicity: **(Nakai *et al.*, 1984)**

Three groups of albino white mice (10 mice in each group) were used for detection the lethal toxicity of *Pasteurella multocida toxin*. First group was injected I/P with 0.2ml of the supernatant. Second group injected I/V 0.2ml of the supernatant. Group three was used as control negative (injected only with saline).

b- Dermonecrotic activity in guinea pigs and rabbits (Nakai *et al*., 1984):

Six groups of Guinea pigs (3 in each group) weighing approximately 300gm/each were used; five groups were depilated laterally on both sides, and then inoculated intradermally (I/D) with a dose of 0.2ml of 2 fold serial dilution with distilled water of the toxin prepared. The reaction was observed at 72hrs post inoculation. Dermonecrotic titers were expressed as the reciprocal of the highest dilution with a positive skin reaction. Group six was used as control negative.

Effect of heating and formalin treatment on the prepared toxin (Nakai *et al*., 1984):

The prepared toxin was heated at various temperatures (37ºC, 70ºC) for 30 min and also treated with 0.5% formalin at room temperature for 1h and 2hrs. The treated samples were examined for its toxicity in mice and guinea pigs.

Six group of white mice (5 mice/group) were used; first group injected with 37ºC heated toxin and the second with 70ºC heated toxin; 3rd group injected with toxin treated by formalin for 1h and the forth group injected with toxin treated with formalin incubated for 2hrs. Fifth group remained as negative control.

Immunization of mice and guinea pigs:

Ginea pigs:

One ml of formalized crude dermonecrotic toxin (10mg toxin /ml incubated for 4 days) with equal volume of complete Freund`s adjuvant, were injected S/C twice per week with 3 days intervals. (**Nakai *et al.*, 1984).**

Studying the immunization effect of prepared toxin (Nakai *et al.*, 1984):

In mice:

Two groups of white mice (10 mice in each group), the first group was immunized (0.5ml S/C) by formalized treated toxin (incubated for 4 days), (**Nakai *et al.*, 1987).** Mice were challenged I/P with 0.2ml of the supernatant (prepared toxin). The lethal effect of toxin was recorded for one week. The second group was divided into 2 subgroups (5 mice in each subgroup), subgroup 2a was injected I/P with 0.2ml of prepared toxin and the subgroup 2b was kept as negative control.

In Ginea pig:

Three groups ginea pig (3 in each group), the first group were immunized (0.5ml S/C) by formalized treated toxin twice a week and at the end of the 1st week ginea pig were challenged I/D with 0.2ml of the supernatant (prepared toxin). The second group was injected I/D with 0.2ml of prepared toxin and the 3rd group was kept as negative control. The dermonecrotic activity of the toxin was recorded for three days after challenge.

Results

Gross lesions of animals died with respiratory infection:

The examined animals revealed that edema of head, neck and inter-mandible region, trachea and lungs were congested, petechial hemorrhages were present throughout the body and lymph node, particularly in the pharyngeal and cervical lymph nodes, these nodes were swollen. Thoracic and abdominal cavities contained blood–tinged fluid. Scattered petechial hemorrhages were visible in the tissues, kidneys were swollen and hyperemic. Liver seemed to be normal. There were petechial hemorrhages on

the pericardium.

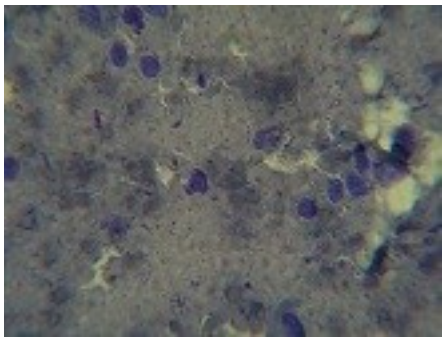
P.multocida was isolated with a percentage of 78.04 % and 32.46 % from nasal swabs of diseased and apparently healthy calves ones respectively Table (1). The bacteriological investigations of the internal organs from the dead calves revealed the isolation of *P.multocida* with percentage of 82.14 % from the collected organs. Table (2)

Microscopically examination of smear slides from dead animals as well as clinical and apparently healthy cases affected with *P.multocida* revealed the typical morphological characteristics of *P.multocida* (Photo. 1).

Table (1). Recovery of *P.multocida* from nasal swabs of clinical cases and contact apparently healthy calves.

Animal status	Type of sample	Total number of samples	Number of *P.multocida* isolates	Percentage (%)
Clinical cases (respiratory infection)	Nasal swabs	123	96	78.04
apparently healthy calves	Nasal swabs	77	25	32.46
Total		200	121	60.5

A



B

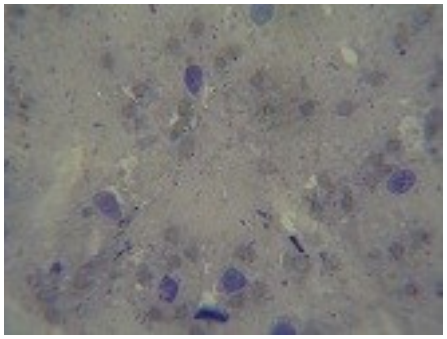


Photo (1). Microscopically examination of smear slides from dead animals (A) as well as clinical and apparently healthy cases (B) affected with *P.multocida* showed coccobacilli stained bacteri**a.**

Table (2). Recovery of *P.multocida* from the internal organs of calves had a history of respiratory infection.

Animal status	Type of samples	Total No. of samples	Number of *P.multocida* isolates	Percentage (%)
Dead animal with respiratory symptoms and fever (14 animals)	Trachea	14	13	*92.85*
	lung	14	12	*85.71*
	Lymph node	14	10	*71.42*
	Liver	14	9	*62.28*
	Peritoneal fluid	14	12	*85.71*
	Pericardial fluid	14	13	*92.85*
Total		84	69	82.14

Congo red binding of *P. multocida*:
All the isolates showed binding to Congo red which appeared as brick red colored colonies of *P. multocida* isolates.

The pathogenicity of isolated *P.multocida:*

In rabbit:
The lethal effect of *P.multocida*: All the inoculated rabbits died within 12hrs after the inoculation of the organisms, necropsy finding revealed the accumulation of a remarkable amount of fibrinopurulent exudates in the thoracic cavity, necrotic foci in the liver and atrophy of the lymphoid organs and tissues.

In mice
Pasteurella multocida was injected I/P into mice causing septicaemia in less than 30hrs.The gross pathology of the disease in mice was characterized by spleenomegaly, lymphadenopathy and petechial haemorrhages (Photo 3).

The toxicity effect of prepared *P.multocida* toxin:
The toxicity of the toxin showed large zone of necrosis more than 5mm in diameter at toxin titer 64 and 32 (100% showing the necrotic zone) and less than 5mm diameter (3.5-4mm) at toxin titre16 and 8 (100 and 66.6% respectively showing necrotic zone) Photo. (2) and Table (3). The highest dermonecrotic toxin titer was 64 followed by the titer 32.

The lethal effect: All mice died when toxin was injected I/P (100%) while 80% died when toxin was injected I/V. The gross pathology was similar to that observed in the pathogenicity of isolated *P.multocida* strain (septicaemia, spleenomegaly, lymphadenopathy and petechial haemorrhages). Photo (3) and Table (3).

Table (3). The dermonecrotic activity *P.multocida* prepared toxin in Guinea pigs.

Groups	No. of animals	titer of toxin	zone of necrosis (mm)*	No. of animal showed dermonecrotic activity	Percentage (%)
I	3	64	+++	3	100
II	3	32	+++	3	100
III	3	16	++	3	100
IV	3	8	++	2	66.6
V	3	4	+	2	66.6
VI	3	2	-	0	0
Total	18			13	72.2

: +++ The diameter of necrosis zone is more than 5mm,
++3.5-4.5mm in diameter, less than 3mm and (–) no necrosis.

Table (4). The lethal effect of *P.multocida prepared toxin* in mice

Experimental groups	Total number of group	route of injection	No. of dead mice*	Percentage (%)
Group 1	10	I/P	10	100
Group 2	10	I/V	8	80
Group 3 (control)	10	-	0	0

*: The mice died within 30 hrs

Treatment of toxin with heat and formalin: Treatment with heating at 70 ºC showed inactivation of the prepared toxin while treatment with heating (37ºC) showed no inactivation.

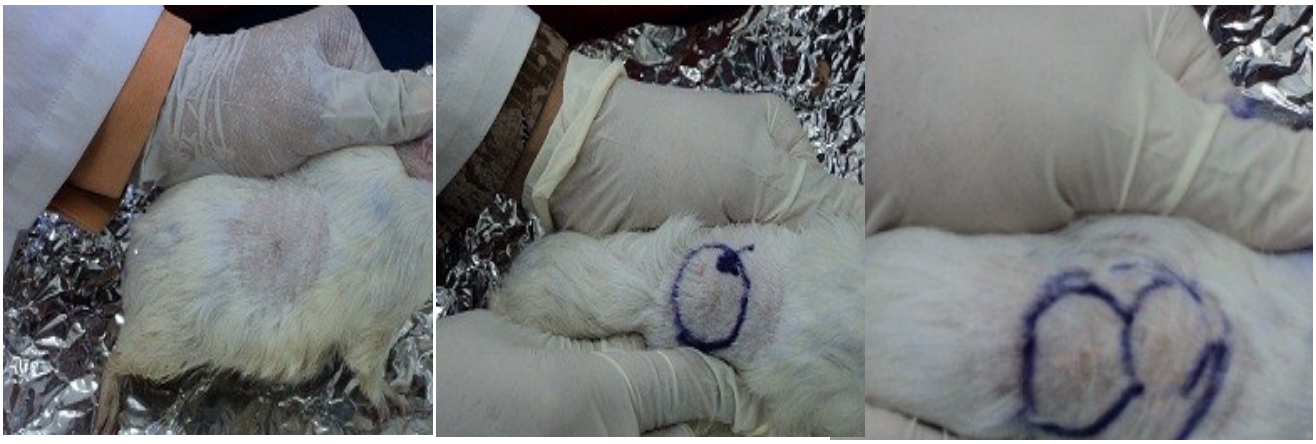


Photo (2). The dermonecrotic activity of *P.multocida* toxin in Ginea pigs

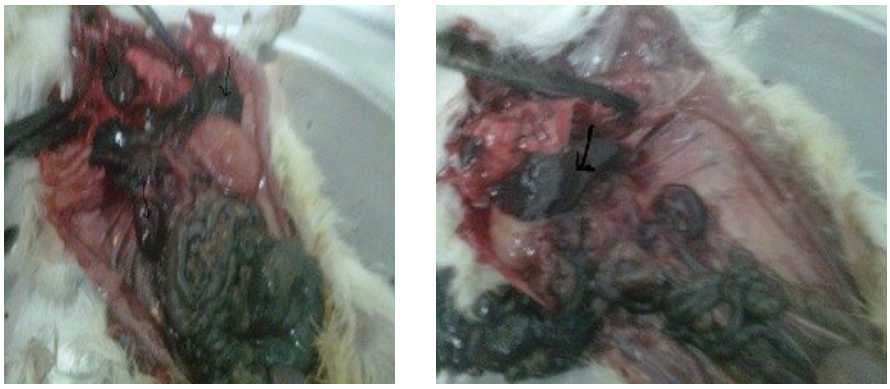


Photo (3). Pathogenicity of *P.multocida* and lethal effect of toxin showing septicemia with petechial hemorrhages in mice (A) and spleenomegaly (B).

The immunization effect of prepared toxin:
In mice: There is no lethal effect in Group 1 (100% survived), while all none immunized Subgroup (2a) died within 30hrs. All Subgroup (2b) still survived as non-challenged control. Table (5)

In ginea pig: The necrotic activity was not observed on group 1 which immunized with the formalized toxin (100%), while all none immunized group 2 showed the necrotic zone. (Table 6)

Table (5). Result of immunization of mice with formalized treated toxin.

Groups	No. of mice/ group	Survived mice		Dead mice	
		N0.	%	No.	%
Group (1)	10	10	100	0	0
Group (2)	10				
Subgroup (2a)	5	0	0	5	100
Subgroup (2b)	5	5	100	0	0

Table (6). Result of immunization of Ginea pig with formalized treated toxin.

Groups	No. of g.pig/ group	No. of g. pig showed dermonecrotic lesion		No. of g. pig with no dermonecrotic lesion	
		N0.	%	No.	%
Group (1)	3	0	0	3	100
Group (2)	3	3	100	0	0
Group (3)	3	0	0	3	100

Discussion

Pasteurella multocida is a pathogenic Gram-negative bacterium that has been classified into three subspecies, five capsular serogroups and 16 serotypes. *P.multocida* serogroup A isolates are bovine nasopharyngeal commensals, bovine pathogens and common isolates from bovine respiratory disease (BRD), both enzootic calf pneumonia of young dairy calves and shipping fever of weaned, stressed beef cattle **(Dabo *et al*. 2007).**

Hemorrhagic septicemia is an acute, fatal, septicemic major disease of cattle and buffaloes **(Farooq *et al*., 2007 and Tabatabaei *et al*., 2007)** caused by *Pasteurella multocida*. The disease has a major impact on the livestock industry in countries where it causes severe economic losses and is ranked as the most important contagious disease of cattle and buffaloes (**Benkirane and De Alwis, 2002).**

The gross lesions of diseased calves were edema of head, neck and inter-mandible region, trachea and lungs were congested, petechial hemorrhages were present throughout the body and lymph node, particularly in the pharyngeal and cervical lymph nodes, these nodes were swollen. Thoracic and abdominal cavities contained blood–tinged fluid. Scattered petechial hemorrhages were visible in the tissues, kidneys were swollen and hyperemic. Liver seemed to be normal. There were petechial hemorrhages on the epicardium. These gross lesions were observed also by **Carter, and De Alwis, (1989)** and **Khan *et al.* (2011)**.

The microscopical examination of affected tissue in diseased and apparently healthy calves showed the stained intracellular coccobacilli. **Mohamed, (2008)** recorded that *P.multocida* were found to be located intracellularly when examined via transmission electron microscope and *P. multocida* cells were visualized in membrane-bound vacuoles within the cytoplasm of the amoebal cells.

All the isolates showed binding to Congo red media which appeared as brick red colored colonies of *P. multocida* isolates. This result was similar to that observed by **Jain (2004)**.

The lethal effect of *P.multocida* and its prepared toxin in all the rabbits died within 12hrs after the inoculation of the organisms and the post mortem lesion observed as well as in mice was similar to that observed by **Rozengurt (1990), Staddon (1991)** and **Higgins (1992)**, who attributed that the lethal effect in rabbits and mice to the production of toxin which stimulated phospholipase C, lead to the activation of protein kinase C, an increase in inositol phosphates, and a rise in intracellular calcium leading to the most common symptoms of respiratory infection and manifested as a nasal discharge. Others include sneezing, congestion, conjunctivitis, clogged tear ducts and intravascular coagulation.

Also, **Horadagoda *et al.* (2001)** reported that *P.multocida* produced endotoxins which are responsible for all manifestations of the disease; these endotoxins have severe effects on central circulation and lead to hypovolaemia (decrease in blood volume). So, fluid administration is the basal stone for the management of infected animals suffering from septicemia**.**

Pasteurella multocida toxin (PMT) or dermonecrotic toxin is produced by capsular type A or D of *P. multocida* **(Peterson, 1990)**. This

toxin can affect the signal transduction pathway of many cells by selective transient activation of a G-protein known as Gqα. Thus, accurate method to detect toxigenic *P. multocida* is needed for improved clinical diagnosis, farm biosecurity, and epidemiological studies as the toxigenic and non-toxigenic *P. multocida* isolates cannot be differentiated by morphology or standard biochemical reactions.

Septicaemia, spleenomegaly, lymphadenopathy and petechial hemorrhages were observed in mice infected with *P.multocida* and its toxin was similar to that observed by **Ramadani, *et al.* (1990)** who reported also that mouse would seem to provide an ideal tool by which to study HS.

In the present study, g.pig injected I/D showed dermonecrotic activity at site of injection, 100% of the infected g.pig showed this activity on 64 and 32 dilution of the prepared toxin. This result was observed by **Bisgaard, (1993); Ackermann *et al.* (1994)** and **Donnio *et al.* (1999),** who reported that *P. multocida* infection lead to local dermatonecrosis, respiratory disease in cattle and rabbit, where toxin binds to a ganglioside-type cell surface receptor.

Complete inactivation of toxin was observed when treated with formalin, while treatment with heating (37ºC) showed no inactivation of the prepared toxin, this results was also observed by **Nakai *et al.* (1984)**.

By studying the immunization effect of prepared dermonecrotic toxin revealed preparation of antitoxin using formalin (0.5%) for 1hrs is immunogenic for the lethal effect of the toxin in mice and for its dermonecrotic activity in g.pig. these results agreed with **Hoda and Eman (2003).**

Conclusion

It can be concluded from this study that HS is highly fatal disease, can provisionally be diagnosis based on a combination of clinical signs, and gross pathological lesions.

A toxigenic strain of *P. miultocida* has been emphasized as a causative agent for haemorrhagic septicemia. Dermonecritic toxin plays an important role in the pathogenesis of *P.multocida* which revealed that identification of toxigenic strain is important as well as preparation of local immunogenic antitoxin can help in prevention of the HS disease.

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بعض الدراسات عن الباسترلا مالتوسيدا المفرزة للسموم فى العجول

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الملخص العربى

تسمم الدم النزفي (HS) هو مرض حاد جداً قاتل، مرض التسسمم فى الأبقار والجاموس واحد من أمراض الماشية الأكثر أهمية اقتصاديا. فهو يحدث بسبب بعض أنواع الباستيريلا مالتوسيدا. ولقد جمعت عينات مجموعها (284)، 200 عينه من المسحات الانفيه للحالات السليمه ظاهريا والحالات التي تعاني من اعراض تنفسيه و84 عينه ا من الأحشاء الداخلية (الرئة، الكبد، والقصبة الهوائية، والعقد الليمفاوية، السائل البريتوني والتامور). وقد تم تسجيل الصفة التشريحية للحيوانات النافقة بأعراض تنفسية وتم مناقشتها.ولقد تم عزل الباستريلا مالتوسيدا بنسبة 78.04% و 32.46% من المسحات الانفية للعجول السليمة ظاهريا والعجول المصابه باعراض تعلى التوالى و82.14 % من الأحشاء الداخلية للعجول النافقة.

ولقد تم دراسة امكانية الباستيرلا مالتوسيدا فى احداث المرض عن طريق طريق تسجيل تأثيرها المميت فى الأرانب والفئران، حيث نفقت كل الأرانب خلال 12 ساعة والفئران خلال 30ساعة ولقد تم تسجيل الصفة التشريحية لهم ومناقشتها.

تم تحضير السم من الباستيرلا مالتوسيدا عن طريق تكسير الخلايا، وقد تم دراسة تأثيره السمى عن طريق تسجيل تأثيره المميت فى الفئران ، وامكانية تنكرز الخلايا عند منطقة الحقن فى الأرانب الهندية. وكان حجم التنكرز فى حدود 4-5 ملليمتر عند تركيز64 ،32.

تم دراسة استخدام السم المعامل بالفورمالين للتحصين قى الفئران والأرانب الهندية، وقد وجد ان ليس هناك نفوق فى المحصنة بنسبة 100% وكذلك ليس هناك تنكرز فى الأرانب الهندية المحصنة بنسبة 100%.