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BACTERIAL AND MOLECULAR CHARACTERIZATION OF PASTEURELLA MULTOCIDA IN INFECTED TURKEYS

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SUMMARY

The primary objective of the study reported here was to evaluate the effect of natural affection of *P.multocida* in Turkeys.The bacteriological examination of five turkey herds in Giza and Beni Swef governorate was done to isolate and identify Pasteurella spp with special attention to *P.multocida.* Outer membrane protein (OMP) profiles of obtained field isolates were done to specify the strain with reference to protein markers (17.5-16.5 kda). The wasting effect of Pasteurellosis on turkey was also determined by comparing the total protein gm % in the turkey organs in each of healthy and pasteurellosed birds. Bacteriological examination showed that 154 out of 400 turkey organs were Pasteurella positive. 62 of them were *P.multocida.* Otherwise, 48.5% of blood serum samples were positive for pasteurella and 18 of these were idintifed biochemically as *P.multocida. Antibiogram* studies of the isolated *P.multocida* indicated that colistin sulphate, trimethopim, sulphamethoxacin,tetracycline, amoxycillin and enrofloxacin were the most effective drugs in vitro. The electrophoretic analysis of the outer membrane protein (OMP) of the *P.multocida* isolates by using SDS-PAGE revealed that all *P.multocida* strains were nearly at 52 kd & 49 kd , where *P.multocida* of group I, II, III were 5.

INTRODUCTION

Fowl cholera (FC) caused by the gram negative bacteria *P.multocida,* has been recognized as an important disease in domestic poultry for more than 200 years (Rosen, 1971; Blackall and Miflin, 2000) FC is a common and widly distributed disease of poultry and has a major economic importance (Rhoades et al., 1989; Jarvinen et al.,

2000). While all species of birds are affected, turkeys are particularly susceptible to pasteurellosis (Rhoades et al., 1989; Rhoades and Rimler 1991). FC can cause devastating economic losses to the poultry industry (Carpenter et al., 1988; Rimler and Rhoades, 1989 A & B). Pasteurellosis is a bacterial disease cause mortality may reach to 20% in adult birds, septicemia, weakness in affected birds, decomposition in turkey meat, bad meat quality and meat intoxication (Thronton and Gracey, 1974). Chronically infected turkeys have been suspected as the major source of infection for susceptible birds (Rhoads and Rimler, 1984). It is critical to determine whether commercial turkeys can be asymptomatic or convalescent carriers of P.multocida (Aye et al., 2001). The infected turkeys with *P.multocida* were suffered from decrease in serum total protein (Friedlander and Olson, 1995; Nixy and Grey, 2000), and body weight (Nestor et al., 1999A; Nixey and Grey, 2000). *P.multocida* is highly pathogenic strain of Pasteurella spp. to the infected turkeys (Joshi et al., 1997). Pasteurellosis in turkeys characterized by emaciation and low meat quality (Nixey and Grey, 2000). Hurber et al., (2002) isolated 66 clinical isolates of *P.multocida* from a total of 100 FC affected birds. Fegan et al., (1995) isolated 42 Pasterurella isolates, from turkey farm and they identified 8 of them as *P.multocida* .Christensen et al., (1998) examined 50 samples from two outbreaks in Danemark and they isolated 30 pasteurella isolates and identified 15 isolates of them as *P.multocida*. Abd-alla (2000) found that *P.multocida* were isolated from 4% of apparently healthy turkeys vs. 9.5% of diseased turkeys.A total number of 95 field *P.multocida* were isolated from 120 different by Guna wardana et al., (2000). Minflin and Black all (2001) isolated 144 Pasteurella isolates from a turkey farm and they identified only two strains as *P.multocida* by RNA and PCR assay. Marandi et al., (1997) studied the antigenicity and geographic origin of 8 *P.multocida* strains isolated from turkeys on basis of electrophoretic mobility of outer membrane protein and polymerase chain reaction (PCR). Aye et al., (2001) recovered 32 *P. multocida* isolates out of 105 turkeys belonging to out break farms vs. no isolates were obtained from either history-outbreak and non-outbreak farms. They also characterized the obtained *P.multocida* via capsular and somatic serotyping, biotyping, restriction endonuclease analysis. They also performed antimicrobial susceptibility testing on all recovered *P.multocida* isolates.Antibiogram studies on *P.multocida* were also performed by Abd-Alla (2000), Olson et al., (2002) and Shivachandra et al., (2004). We used electrophorotic analysis outer cell layer protein of *P.m altocida* for confirm its profile isolation and identification sample (Rossmanith et al., 1991). Thus the objectives of this study were isolate and identify the field strains of pasteurella species with special reference to *P. multocida* in commercial turkey farms belonging to Giza and Beni sweef governorates. Antimicrobial susceptibilities of these *P. multocida* isolates were determined, Also detec-

tion of the total protein ratio in the serum was performed to determine the deteruration of the turkey meat quality. And finally characterization of the *P.moltucida* strains was done by using their whole outer cell protein extracts and SDS-PAGE technique.

MATERIALS AND MATHODS

The present investigation was carried out on birds of five turkey farms in Giza and Beni Swef governorates. A total of 50 birds, ten belong to each farm, were used for sampling. Whereas, individual organs including liver, spleen, lung, gizzard, thigh and breast were aseptically sampled for bacteriological and further molecular examinations. Blood serum samples were aseptically and individually obtained from the aforementioned 50 birds as well as from additional 50 birds (ten from each turkey farm) for investigation. All samples were obtained during the antimortum examination in different farms in Giza and Beni swef governorates.

Bacterial isolation :

Samples were inoculated on nutrient broth and tryptos soya broth then incubated at 37°C for 24 hrs and, subcultured on blood agar, mackonkey agar and incubated at 37°C for 24 - 48 hrs. Suspected colonies were picked up for morphological, biochemical and physiological characterization (Cruickshank et al., 1975; Kirg and Holt, 1984; Rhoades and Rimler, 1991 and Koneman et al., 1992).

Bacterial identification:

Films from pure cultures of the isolated organisms were stained by Gram's staining technique and examined microscopically for the presence of charactarestic bipolarity feature of pasteurella microorganisms. Suspected pasteurella colonies were subjected to indol , catalase tests and sugar fermentation of Lactose, Maltose, Mannitol, Trehalose and Xylose. In vitro antibiogram test of isolated was carried out according to Quinn et al., (2002).

O.M.P technique:

15 Mg of *P.multocida* outer cell membrane protein electrophoresed using 10% SDS-PAGE under reducing condion (Laemmli, 1970). The fractionated antigens were virulized by silver staining technique. The gel was soaked overneight in the silver reagent. The gel was then destained with destaining solution (45% methanol, 5% glacial acetic acid and 50% destilled water) with several changes, till the bands became clear. Determination of total protein gm% was done by spectrophotometer according to John, (1996). The total protein gm% samples of breast and thigh muscles were estimated by the dividing to 5 groups where each group formed from 5 douple samples (from breast and thigh). We estimat also the total protein gm% in 5 from healthy turkeys breast and thigh. We compare the total protein gm% examined and healthy samples.

Outer membrane protein of *P.multocida* test:

a- Silver strain used:

b-Silver staining:

Table (1)

T 1

Step	Solutions	Time	
Fixation	Trichloroacetic acid (TCA) Make up to 250 ml with distilled water	50g	30 min
Washing	Distilled water		3x5 min
Sensitizing	Ethanol Glutardialdehyde (25% w/v)* Sodium thiosulphate (5% w/v) Sodium acetate (17g) Make up to 250ml with distilled water	75ml 1.25ml 10ml 1packet	
Washing	Distilled water		3x10 min
Silver reaction	Silver nitrate solution (2.5% w/v) Formaldehyde (37% w/v)* Make up to 250 ml with distilled water	25 ml 0.1 ml	20 min
Washing	Distilled water		2x1 min
Developing	Sodium carbonate (6.25g) Formaldehyde (37% w/v)* Make up to 250 ml with distilled water Stir vigorously to dissolve sodium carbonate	1packet 0.05 ml	20 min
Stopping	EDTA-Na_2.$2H_2O$ (3.65g) Make up to 250 ml with distilled water	1paket	10 min
Washing	Distilled water		3x5 min
Preserving for plastic backed gels:	Glycerol (87% w/w) Make up to 250 ml with distilled water	25 ml	20 min
Preserving for gels not supported on plastic films	Ethanol Glycerol (87% w/w) Make up to 250 ml with distilled water	11.5 ml	2x30min

according manufacture company.

RESULTS

Bacterial isolation and indentification:

Out of 400 samples a total of 194 (48.5%) were pasteurella positive. Among the examined organs, liver and spleen recorded the highest isolation rates, while gizzard and breast had the lowest isolation rates. The isolation rates of Pasteurellae were recorded in table 2. Bacterial identification allotted the isolated Pasteurellae into *P.multocida* (41.2 %), *P.haemolytica* (34.0 %) and *P.gallinarum* (24.8 %). Distribution of *P.multocida*, *P.haemolytica* and *P.gallinarum* among the different turkey organs were recorded in table 3. Analysis of cultural, biochemical and physiological characteristics of Pasteurella isolates (Table 4) indicated that 80, 66, 48 out of 194 isolates were *P.multocida*, *P.haemolytica* and *P.gallinarum* respectively. Antibiogram study of the isolated *P.multocida* as shown in table 5 indicated that colistin sulphate, tetraeyclin and trimethoprim / sulphamethoxyin were the most effective drugs,while flumeequin and erythromycin were the lowest efficient ones.The results in table (6) revealed that *P.multoceda* solution were SDS-PAG test in 3 groups at first in liver, lung, spleen and gizzard, The second in breast and thigh and finally in serum. The T (7) & fig.1 revealed the KDa of every strain of *P.multocida* which appears in the bands of SDS-PAG of OMP analysis.

SDS-PAG analysis of OMP of *P.multocida* species revealed that up to 6 protein bands were from KD 15 to 16.5.

The molecular weight of the polypeptide bands estimated by comparison of standard M.W markers (Rainbow, T.M.) run in parallel were of M.W. 175 KDa, 83 KDa, 62 KDa, 47.5 KDa, 32.5 KDa and 16.5 KDa.

OMP profile of *P.multocida* revealed that the 3 group of isolates (one isolate from samples liver , lung, spleen & gizzard, 2nd from breast and thigh sample and the 3rd from serum samples) were examined by SDS- for conferm their isolations. The OMP technique revealed that *P.maltucida* isolated from breast and thigh were presented in lane No.1 but *P.maltucida* isolates from liver and gizzard were presented in lane 2 where as *P.maltucida* which isolated from serum were presented in lane 3 but the marker were represented in lane 4.

Table (2): Isolation of Pasteurella species in the examined samples.

Type of Samples	No. of Examined Samples	Positive	
		No.	%
Liver	50	30	60
Lung	50	26	52
Spleen	50	30	60
Gizzard	50	22	44
Breast	50	22	44
Thigh	50	24	48
Serum	100	40	40
Over all	400	194	48.5

Table (3): Distribution of Pasteurella isolates in the examined samples

Type of Samples	No. of Examined Samples	No. of identified Pasteurella					
		P. multocida		*P. haemolytica*		*P. gallinarum*	
		No.	%	No.	%	No.	%
Liver	30	12	40.0	10	33.3	8	26.7
Lung	26	14	53.8	7	26.9	5	19.3
Spleen	30	12	40.0	11	36.7	7	23.3
Gizzard	22	10	45.5	7	31.8	5	22.7
Breast	22	6	27.3	9	40.9	7	31.8
Thigh	24	8	33.3	10	41.7	6	25.0
Serum	40	18	45.0	12	30.0	10	25.0
Over all	194	80	41.2	66	34.0	48	24.8

Table (4): Differential biochemical and physiological characteristics of the isolated Pasterellae.

Test	{psitive reactors		Negative reactors	
	No/194	%	No/194	%
Hemolysis	66	34.0	128	66.0
Mac Conkey's agar	65	33.5	129	66.5
Indol	80	41.2	114	58.8
Catalase	144	74.2	50	25.8
Fractose	66	34.0	124	66.0
Maltose	50	25.8	144	74.2
Xylose	128	66.0	66	34.0
Trehalose	115	59.3	79	40.7
Mannitol	146	75.3	48	24.7

Table (5): Antibiogram study of isolated *P.multocida*

Antibiotics	Sensitive isolates		Resistant isolates	
	No	%	No.	%
Amoxycillin	72	90	8	10
Ampicillin	64	80	16	20
Chloramphenical	48	60	32	40
Colistin sulphate	80	100	--	0.0
Danofloxacin	48	60	32	40
Erythromycin	--	0.0	80	100
Enrofloxacin	72	90	8	10
Gentamycin	56	70	24	30
Flumequin	--	0.0	80	100
Penicillin	16	20	64	80
Sulphamethoxazol	80	100	--	0.0
Tetracycline	80	100	--	0.0

Table (6): Outer membrane protein (OMP) by SDS-PAGE of *P. multocida* isolated from different samples.

Sample	(OMP) test
Liver, lung, spleen & gizzard	+ ve
Breast, thigh	+ ve
Serum	+ve

Table (7): Plasmid profile of *P. multocida* isolates.

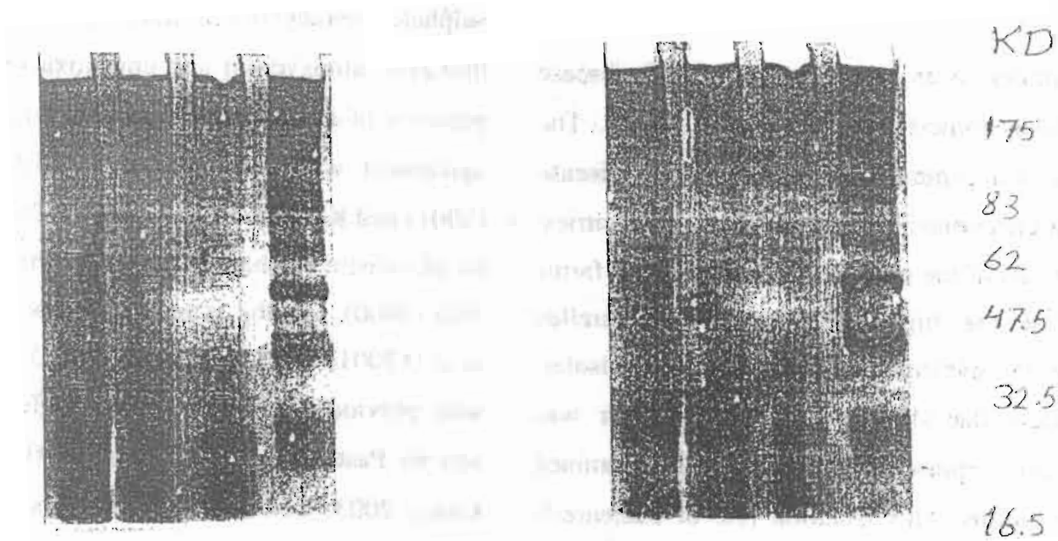
Lanes:	Lane 1	Lane 2	Lane 3	Marker
Rows	(mol. w.)	(mol. w.)	(mol. w.)	175
r1		156.2		83
r2		101.13		62
R3	59.441		58.223	
r4	51.765	52.088	52.618	
r5	49.937	49.45	48.353	47.5
r6	44.052			
r7	41.379			
r8	36.638	34.053		
r9	32.048		31.274	32.5
r10	27.661	29.145	29.274	
r11	25.597		23.597	
r12	21.145	20.113		
r13		17.984	16.5	16.5
r14	15.597	14.823	13.79	
r15	5.9839	5.6613	5.9839	
r16	2.8871	2.2419	3.0806	

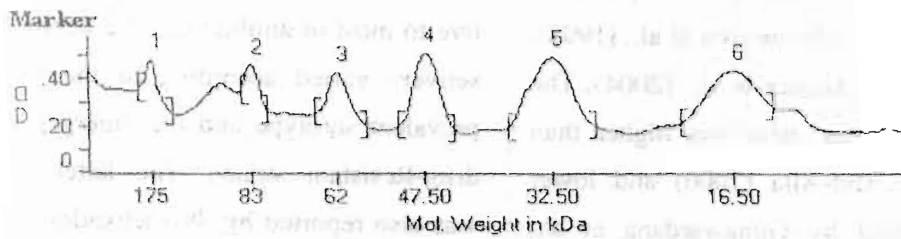
Table (8): Total protein gm% in examined breast samples.

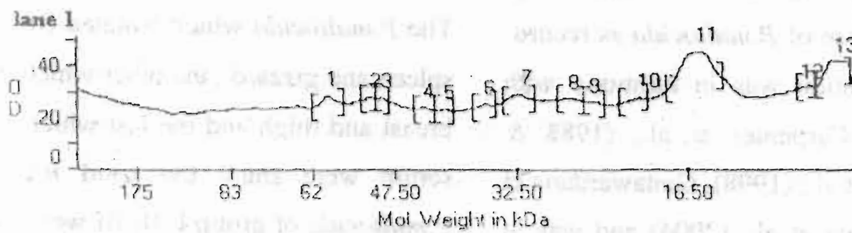
Healthy birds samples	Examined birds				
	Group 1	Group 2	Group 3	Group 4	Group 5
21.9	16.0	17.7	16.0	17.5	17.1
21.7	15.1	16.5	17.1	16.9	15.9
21.8	17.7	16.9	17.5	16.1	15.5
21.7	19.3	17.0	16.5	15.4	15.4
21.6	8.1	17.1	16.5	16.0	15.6

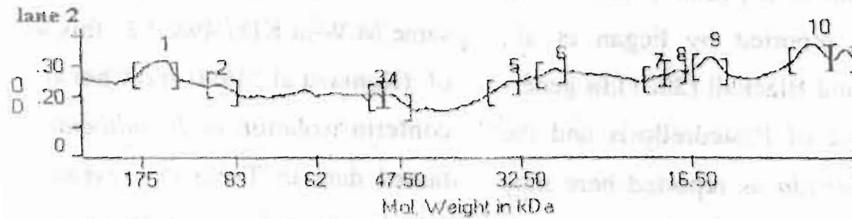
Table (9): Total protein gm% in examined thigh samples.

Healthy birds samples	Examined birds				
	Group 1	Group 2	Group 3	Group 4	Group 5
18.3	14.1	15.5	16.1	13.3	15.1
18.1	15.0	15.4	13.1	13.4	14.5
18.2	16.6	15.7	15.9	14.5	13.9
18.0	17.1	16.0	15.5	15.1	14.0
18.0	14.2	19.9	15.8	13.1	14.7


KD
175
83
62
47.5
32.5
16.5


Marker
OD
175
83
62
47.50
32.50
16.50
Mol. Weight in kDa


lane 1
OD
175
83
62
47.50
32.50
16.50
Mol. Weight in kDa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OD
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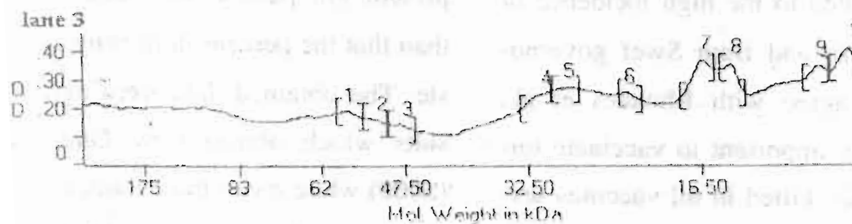
83
62
47.50
32.50
16.50
Mol. Weight in kDa


lane 3
OD
175
83
62
47.50
32.50
16.50
Mol. Weight in kDa

DISCUSSION

Fowl cholera is an acute, fatal septicemic disease of various domestic and wild bird species. The disease is a common, widely distributed disease of major economic importance in many countries. The results of the present work on 5 turkey farms indicated the high incidence of Pasteurellae among the investigated birds . Among the isolated pasteurellae (194 isolates), *P.multocida* was 80 isolates representing 41.2 % of the examined turkey organs. This isolation rate of Pasteurella species were similar to those reported by Thronton and Gracey (1974), Christensen et al., (1998), Aye et al., (2001) and Kumar et al., (2004). The isolation rate as reported here was higher than those reported by Abd-Alla (2000) and lower than those reported by Gunawardana et al., (2000). The prevalence of *P.multocida* as recorded by our identification was in harmony with those reported by Carpenter et al., (1988 & 1989), Christensen et al., (1998), Gunawardana et al., (2000) and Kumar et al., (2004) and was in contrast with those reported by Fegan et al., (1995) and Minflin and Blackall (2001).In general, the high incidence of Pasteurellosis and the prevalence of *P.multocida* as reported here may indicated and attributed to the high incidence of FC outbreaks in Giza and Beni Swef governorates. The authors agree with Rhoades et al., (1989) that it is very important to vaccinate turkey with *P.multocida* killed in oil vaccines several times. The results of antibiogram study as reported here indicated the superiority of colistin sulphate, tetracycline, trimethopin / sulphamethoxazol, amoxycillin and enrofloxacin. The superiority of enrolfoxacin as reported here was in agreement with those reported by Aye et al., (2001) and Knott and Lister (2003). The superiority of colistin sulphate was also reported by Abdo Alla (2000), and the superiority reported by Aye et al., (2001) and Olson et al., (2002).Tetracyclin was previously recorded as an effective drug against Pasteurella (Abdo Alla, 2000; Knott and Lister, 2003; Olson et al 2002 and shivachandra et al, 2004). In general, Pasteurellae were sensitive to most of antibiotics. The degree of its sensetivety varied according to the localities the prevalent serotype and the emergence of multi-drug-Resistant strains. The latter phenomenon was also reported by Shivachandra et al. (2004). The *P.multocida* which isolated from liver, lung, spleen and gizzard , the other which isolated from breast and thigh and the last which isolated from serum were share the band R4 &R5 where *P.multocida* of group I, II, III were simillar at the same M.W at KDa 49& 52 , this was simillar that of (Kania et al., 1990 and Choi et al., 1989). This conferm isolation of *P.multocida* strain the obtained data in Table (8) revealed that the total protein gm percent in breast must were lower than that the percent in healthy turkey breast musle. The obtained data were agreed with the results which obtained by Gunawardana et al., (2000) while mean the obtained data in Table (9) were agreed with those obtained by Li et al.,

(2000), these data revealed that the protein gm percent in the examined turkeys thigh lower than that in healthy turkey thigh muscles. These study indicated that infected turkeys with Pasteurella were suffered from low total protein gm percent, lowmeat quality and the breast, thigh and offals were reservoir of *P.multocida*. The meat and offal of affected turkeys by Pasturella must be bacteriologicaly examined before passing for consumpition. The control measures must be taken to turkey farms for consumers health the turkeys must be vaccinated, this may decrease the ability to infection. The high incidence of pasteurellosis in turkey may indicate importance of vaccinateion programs. Colistin sulphate, Sulphamethoxazol &Tetracycline are the preferable antimicrobial drug in case of pasteurellosis in turkey farms.

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الخصائص البكتريولوجية والجزيئية للباستريلا مالتوسيدا فى الرومى المصاب

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يعتبر ميكروب الباستيرلا من أهم الميكروبات المرضية للرومي مما يسبب خسارة إقتصادية عالية للمربيين والمستهلكين وهو من الأمراض المشتركة التى تسبب ضرر بالغ للإنسان من خلال الميكروب نفسه أو من خلال السموم التى تفرزها، تم تجميع ٣٠٠ عينة من لحوم الرومى وأحشاءه الداخلية وكذلك ١٠٠ عينة من مصل الدم المذبوح بمجازر وبنى سويف وتم فحصهم بكتريولوجيًا لتحديد مدى إصابتها بميكروب الباستيرلا ، أمكن عزل الميكروب الباستيرلا من عينات الكبد ، الرئه ، الطحال ، القوانص، الصدر ، الورك ومصل الدم بنسبة ٦٠٪ ، ٥٢٪ ، ٦٠٪ ، ٤٤٪ ، ٤٨٪ و ٤٠٪ على التوالى والترتيب وتم تصنيف عترة الباستيرلا بيوكيميائيًا إلى باستيرلا مالتوسيدا وكانت نسبة الباستيرلا مالتوسيدا من العترات المعزولة٤٠٪ ، ٣٨.٥٪، ٤٠٪ ، ٤٥.٥٪ ، ٣٣.٣٪ و٥٤٪ على التوالى والترتيب من عينات الكبد ، الرئه ، الطحال ، القوانص ، الصدر ، الورك ومصل الدم. بإستخدام إختبار الحساسية ضد المعزولات المختلفة. كانت أكثر المضادات الحيوية تأثيراً فى الباستريلاما ماتوسيدا هى الكولستين سلفيت ، السلفاميثوكسازول والتراسيلكين، تم إستخدام تفاعل بروتين جدار الخلية الخالية فى تاكيد عزل الباستيرلا الماتوسيدا من جميع العينات وكانت العترات المعزولة تشترك فى الصف ٥٢ كيلو دالتون و٤٩ كيلو دالتون بالمقارنة بلان الماركر ، وتم قياس نسبة البروتين الكلى فى لحوم الصدور ولحوم الوراك وتم مقارنتها بالنتائج الحاصلين عليها من قياس نسبة البروتين الكلى فى لحوم صدور ووراك رومى سليمة صحيا واتضح ان الرومي المصاب بالباستيرلا يعانى من إنخفاض جودة لحومه هذا وقد نوقشت أهمية وخطورة ميكروب الباستيرلا على الصحة العامة وعوامل الخطورة على المزارع وقد نوقشت التوصيات اللازمة التى يجب مراعتها لمنع انتشار المرض فى المزارع وحماية المستهلك.