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STUDIES ON *PSEUDOMONAS* SPECIES IN MILK AND FECES OF DAIRY CATTLE

(With 11 Tables and 3 Fingers)

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دراسات عن ميكروب السودوموناس في حليب وروث أبقار الحليب

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يعد ميكروب *السودوموناس* من الميكروبات ذات التأثير الضار بما يسببه من مشاكل تؤثر علي صحة الإنسان و الحيوان معا. و يعتبر تلوث الألبان بميكروبات *السودوموناس* المسببة للمعايب دلالة علي إهمال الاشتراطات الصحية الواجبة إتباعها إنتاج الألبان في المزارع فضلا عن أن تكاثرها في الألبان قد يؤدي إلى ظهور بعض المعايب التي تؤثر علي جودتها و صلاحيتها للاستهلاك الآدمي. وقد دلت النتائج على تواجد ميكروبات *السودوموناس* في كل من الألبان والروث الناتج من حيوانات تعاني من إسهال بمتوسط بتراوح بين ٩ ×١٠ إلى ٦.٨×١٠ ١٢ و ٦.١×١٠ ٦ إلى ٩ ×١٠ ١٢ على التوالي. وقد تواجد ميكروب *السودوموناس ايروجينوزا* بأعلى نسبة في كل من الألبان و الروث معا. وقد تبين من الدراسة قدرة ميكروب *السودوموناس ايروجينوزا* علي إفراز سموم خارجية وداخلية حيث تم تحديد الجرعة المميتة لكلا منهما والتي أدت إلى وفاة جميع الفئران المحقونة بها. بالإضافة إلى ذلك فقد تم تحضير الأمصال النوعية المضادة لكل من السموم الخارجية والسموم الداخلية في الأرانب واختبار قدرتها على الصد والتحدي. هذا وقد توقشت الأهمية الصحية و الاقتصادية للميكروبات المعزولة و التوصيات الواجب إتباعها لتجنب المخاطر الناتجة عنها.

SUMMARY

In the present study, a total of two hundred and ten samples of milk and feces (one hundred and five each) of diarrheic dairy cows were collected and examined for isolation, identification and toxin production of *Pseudomonas*. The total *Pseudomonas* count ranged from $9x10^3$ to $8.6x10^{12}$ & $1.6 x10^6$ to $9x10^{12}$ cfu/ml with mean values $9.4x10^{10} \pm$

$1.1x10^8$ and $7.5 x10^{11} \pm 3 x10^6$ cfu/ml in milk and feces, respectively. *P. aeruginosa* was the most predominant species isolated from the examined milk and fecal samples. *P. aeruginosa* exotoxin and endotoxin were extracted and identified by electrophoresis. Lethal Dose Fifty (LD_{50}) of exotoxin and endotoxins varied from 25 to 100 µg and 2500 to 3000 µg, respectively. All mice that were inoculated I/V by produced exotoxins or endotoxins died within three days post inoculation. Protective antisera against the obtained exotoxins and endotoxins were prepared in rabbits. It was concluded that *P. aeruginosa* serovars play an important role among diarrheic dairy cows and is indication for potential hazard associated with milk quality.

Key words: *Pseudomonas, Milk, Feces, Dairy Cattle.*

INTRODUCTION

In recent years, there is a great interest directed to the role of opportunistic Gram negative psychrotrophs in dairy animals. Particular attention has been directed to *Pseudomonas* infection because of its pathogenicity to human and domestic animals. *Pseudomonas* species are widely distributed in nature, they have been found in external environmental conditions surrounding dairy animals such as water, soil, sewage, air, grass, hay, feces and bedding materials. These organisms represent the most common psychrotrophs that contaminate milk and cause variety of defects including fruity, rancid, bitter and putrid flavor as well as color defects (Kraft, 1995 and Pearson et al., 2000).

P. aeruginosa is considered all over the world as one of the dangerous organisms causing different diseases and capable of secreting many extracellular products such as lipopolysaccharide (LPS), fibrinolysin, hemolysins, exotoxins and enterotoxins. These products have a major role in the virulence of pathogenic strains of *P. aeruginosa* (Champagne et al., 1994 and Beuchat, 2000). Pathogenic strains of *P. aeruginosa* play an important role in mastitis, endometritis, chronic pulmonary diseases, urogenital tract infection, cystic fibrosis and severe forms of gastroenteritis among man and animals (Osman et al., 1998 and Ribas et al., 2000).

With the extensive use of refrigerated storage in the dairy industry, the significance of *Pseudomonas* species particularly *P. aeruginosa* in the spoilage of raw milk and other dairy products has increased dramatically. The growth of these organisms in raw milk is of

particular importance due to production of proteinase, lipase, phospholipase C and glycosidase enzymes strongly damaging milk fat protein membrane which reflect on the quality of the finished products including cream, butter and cheese (Suhren, 1995 and Jayarao and Yung, 1999). *Pseudomonas* species can be eliminated by pasteurization or UHT treatment, but their enzymes are able to resist heat treatment used for processing of raw milk and have been implicated in spoilage of ultra heat treated milk and other dairy products (Dieckelmann et al., 1998 and Lira and Nielsen, 1998).

Therefore, due to the increase interest of *Pseudomonas* species particularly *P. aeruginosa* as health risk affecting human and animal, the present study aimed to study the following items:

- Isolation and identification of *Pseudomonas* species from milk and feces of diseased dairy cows.
- Screening of the isolated species for exotoxin and endotoxins production.
- Purification and estimation of the obtained toxins using SDS-PAGE.
- Studying the lethality, immunogenicity and protective effect of the recovered toxins in mice.

MATERIAL and METHODS

1- Bacteriological examination:

Sampling:

A total of two hundred and ten samples of milk and feces (one hundred and five each) were collected from Friesian dairy cows showing diarrhoea among several dairy farms in Giza Governorate. The collected samples were transferred to the laboratory in icebox with a minimum of delay to be examined at the Department of Food Hygiene, Faculty of Veterinary Medicine, Cairo University.

Isolation of *Pseudomonas* species:

Bacteriological swabs of the milk and fecal samples were inoculated into sterile nutrient broth and incubated at 37°C for 24h. At the end of incubation, a loopfull from each tube was streaked onto the surface of pseudosel TM agar (BBL) and blood agar plates and incubated at 37°C for 24 hours. Total *Pseudomonas* count in milk and fecal samples were performed according to Collins et al., (1995).

Identification of isolates

The suspected isolates were purified and identified according to Koneman et al., (1992) and Quinn et al., (1994). Typing of the *P.*

aeruginosa isolates were performed by slide agglutination test using *P. aeruginosa* specific antisera polyvalent I (A, C, H, I & L group), polyvalent II (B, J, K & M group) and polyvalent III (D, E, F, G & N group) obtained from Denka Seikenco., Tokyo, Japan.

2. Extraction and purification of *Pseudomonas* toxins

- Extraction and purification of exotoxins and endotoxins from *P. aeruginosa* isolates were performed according to Qureshi and Takayama, (1982) and Liu et al., (1996).
- Exotoxin and endotoxin preparations were assayed for their purity by SDS PAGE according to Sambrook et al., (1989).
- Staining of the toxins in gel were carried out according to Tsai and Frasch, (1982).

3. Toxin assay

Mouse lethality bioassay was used to measure toxicity of the obtained toxins in Swiss white mice. The mortality rate was recorded for 5 days. Lethal dose fifty (LD_{50}) values were calculated as described by Lynn and Collahan, (1976).

4. Immunogenicity of the obtained toxins

According to Gresiman and Johnson (1988) and Liu et al., (1996) specific antibodies against the obtained exotoxins and endotoxins were prepared in rabbits. Antibody level against *P. aeruginosa* in the sera of rabbits was titred by passive haemagglutination test .

5. Protection study in mice

The protective activity of the prepared antibodies was assessed by I/V inoculation in mice. After 1-2 hours, these mice were challenged by I/V injection with LD50 of its corresponding toxin, as well as *P. aeruginosa* serovars according to Gresiman and Johnson, (1988).

RESULTS and DISCUSSION

The results presented in Tables (1&2) revealed that *Pseudomonas* species were identified in 48.57% (51/105) & 51.43% (54/105) of milk and fecal samples respectively. The total *Pseudomonas* count ranged from $9x10^3$ to $8.6x10^{12}$ & $1.6 x10^6$ to $9x10^{12}$ cfu/ml with mean values $9.4x10^{10} \pm 1.1x10^8$ and $7.5 x10^{11} \pm 3x10^6$ cfu/ml in milk and feces respectively. The highest frequency distribution (23.53% & 31.48 %) lies within the range of 10^9-10^{10} in the examined milk and fecal samples.

Nearly similar findings were reported by Ternstrom et al., (1993); Jayarao et al., (1997) and Uraz and Citak, (1998).

The recommended guide line for evaluating *Pseudomonas* count in milk, which was published by Jayarao et al., (1997) and Jayarao and Yung (1999), suggested that milk contain more than 10^7 cfu/ml of poor quality. Therefore, about 50.98% of the examined milk samples, had greater than 10^5 cfu/ml, are considered as low grade milk (Barmley and McKinnon, 1990).

Pseudomonas species identified from the examined milk and fecal samples were represented by *P. aeruginosa* (27.45 & 31.48%); *P. alkaligenes* (23.53 &29.93%); *P. putida* (21.57 & 22.22 %); *P. aurefaciens* (15.69 &12.96%); and *P. mesophilia* (11.76 &7.41%) respectively (Table 3 & Fig 3).

Nearly similar finding were reported by Mickova et al., (1989); Ternstrom et al., (1993); McKay et al., (1995); Szita et al., (1998) and Uraz and Citak, (1998).

As shown in Tables (3&4), *P. aeruginosa* was the most predominant identifiable species isolated from the examined milk and fecal samples. The most common serotypes of *P. aeruginosa* detected in milk and feces were poly III D 42.86% (6/14) & 35.29% (6/17) and poly III G 21.43% (3/14) & 23.53% (4/17) while poly II B was zero & 11.76% (2/17) respectively. These findings agreed to a certain extent with Schildger et al., (1989). The high prevalence of *P. aeruginosa* in milk of diarrheic dairy cattle may be due to the contamination of milk by fecal matter during milking or their shedding in milk constantly or intermittently (Rebhun et al., 1995 and Dieckelmann et al., 1998). This organism responsible for milk spoilage (off odor and bitter flavor) due to breakdown of amino acids. The onset of amino acids degeneration takes place when relatively low number of *Pseudomonas* organisms present (10^6/ml). When the numbers of *Pseudomonas* in milk reach 10^7/ml or above, significant amounts of lipase and proteinase enzymes are produced. These enzymes show high heat resistance and give rise to problems in the manufactured milk as rancidity of cheddar cheese, casein breakdown in UHT milk, slow cheese making and reduction of cheese yields (Suarez and Ferreiros, 1991; Lira and Nielsen, 1998 and Jayarao and Yung 1999).

The obtained results revealed that, milk from diseased dairy cattle should be examined not only to determine milk quality but also to detect pathogens of animal health significance such as *P. aeruginosa*. It is considered as an important agent in dairy cows, reflecting potential problems associated milk quality due to their production of lipase and proteinase enzymes and public health hazard by their exotoxin and

lipopolysaccharide endotoxins production (Adlard et al., 1998 ; Jayarao & Yung 1999 and Yu and Martin, 2000).

P. aeruginosa produces number of toxic substances that have been implicated in the pathogenicity of the organisms. When the extracted exotoxin subjected to SDSPAGE, appeared to contain from eight to six protein components migrate during electrophoresis at similar rates within a close range of molecular weight approximately 50 to 71KD (Table 5 & Fig 1). The obtained results agreed with those reported by Lynn and Collahan, (1976) who indicated that the exotoxins were larger than ovalbumin which has molecular weight of 50 to 55 KD.

Results recorded in (Table 6 & Fig 2) indicated that major bands of endotoxin in SDSPAGE were detected after staining with silver nitrate method representing the core region with low molecular weights varying from 7 to 12 KD. Nearly similar finding were reported by Gennari and Dragotto, (1992).

The results presented in Tables (7&8) revealed that there was a wide variation in LD_{50} values between exotoxin and endotoxins extracted from *P. aeruginosa*. LD_{50} values varied from 25 to 100 µg and 2500 to 3000 µg for exotoxins and endotoxins respectively. This results run parallel to those obtained by Callahan, 1976 and Bjorn et al., (1977) who reported that endotoxin was approximately a thousand fold less lethal for mice than exotoxins.

All mice inoculated I/V by exotoxins and endotoxins of the isolated *P. aeruginosa* serovars died within three days post inoculation (Table 9). The onset of lethality after administration of endotoxins was as fast as 24 hours compared to 24-72 hours with exotoxin inoculated mice. Hypothetically exotoxin could disturb the host immune system by breaking down the normal anatomical defense barriers and interfering the leukocyte function. In natural infection exotoxin may enhance the invasiveness of *Pseudomonas* increasing bacteremia, or interfere with normal clearance mechanisms (Pollack et al., 1977). On the other hand, lipopolysaccharide endotoxin (LPS) when given I/V produced a toxic shock causing hypothermia, damage to membranes and coagulation of blood through complement C activation (Mergenhagen et al., 1972 and Liu et al., 1996).

Rabbits immunized with exotoxin preparation showed titres ranged from 1/640 to 1/ 2500 (Table 10). There was no sharp differences detected in antibody level between *Pseudomonas* isolated from milk and those from feces. The antibody obtained in the present study was similar to that reported by Liu and Hsieh, (1973). Immunization of extracted

endotoxin preparation in rabbit showed reduction of antibody titres in immunized rabbit from 1/320 to 1/640. These results came in point with Gresiman & Johnson, (1988) and Elsheith et al., (1988). This indicated that, exotoxin is more potent than Lipopolysaccharide (LPS) endotoxin.

The protective capacity of the obtained rabbit hyper immune sera against homologus exotoxin of *P. aeruginosa* serovars were estimated using passive mice protection test (Table 11). The results showed that, exotoxin antibodies were 90- 100% protective against the challenge with its homologus exotoxin while it gave 60 to 80% when challenged with the *Pseudomonas* serovars. The obtained results were similar to that obtained by Pavlovskis et al., (1977); Zschusk and Schleisser, (1986) and Grover et al., (1990) who concluded that exotoxins of *Pseudomonas* is an important pathogenic factor.

When passive mice protection test carried out to estimate the protective capacity of endotoxin antibody in challenge with homologus endotoxin and homologous *Pseudomonas* serovars. the results showed 90-100% protection. These results agreed to a certain extent with that reported by Greisman and Johnson (1988). Endotoxin antibody was not effective in protecting against *pseudomonas* serovars (20-30%). These results agreed with those reported by Lui and Hsieh, (1973) and Elsheith et al., (1988) who concluded that LPS somatic antigen was not important factor in pathogenesis. It is hoped that the obtained data will lead to more study about the pathogenesis and control of *Pseudomonas* in dairy cattle.

In conclusion, it was clear that *P. aeruginosa* serovars plays an important role among diarrheic dairy cows. As well as, the effective protection of the specific hyperimmune serum against exotoxins and endotoxins (LPS) produced by *P. aeruginosa*. Regarding the high count of *Pseudomonas* species especially *P. aeruginosa* and their toxins released in milk, efforts should be applied to minimize milk contamination by application of strict hygienic measures during milk production, processing, distribution and storage. Moreover, infection elimination procedures should be carried out among dairy cows especially those suffering from diarrhea.

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Table 1: Statistical analytical results of total *Pseudomonas* count/ml of the examined milk and fecal samples

Samples	Total No.	Positive	%	Min	Max	Mean	S.E.M. ±
Milk	105	51	48.57	$9x10^3$	$8.6x10^{12}$	$9.4x10^{10}$	$1.1x10^8$
Feces	105	54	51.43	$1.6x10^5$	$9x10^{12}$	$7.5x10^{11}$	$3x10^6$

Table 2: Frequency distribution of examined milk and fecal samples based on their total *Pseudomonas* count.

Frequency	Milk		Feces	
	Positive	%	Positive	%
10^3-10^4	4	7.84	-	0.00
10^4-10^5	6	11.76	-	0.00
10^5-10^6	5	9.80	5	9.26
10^6-10^7	4	7.84	2	3.70
10^7-10^8	6	11.76	8	14.81
10^8-10^9	8	15.69	10	18.52
10^9-10^{10}	12	23.53	17	31.48
10^{10}-10^{11}	4	7.84	12	22.22
10^{11}-10^{12}	2	3.92	3	5.56
Total	51	100	54	100

Table 3: Incidence of *Pseudomonas* species isolated from the examined milk and fecal samples

Type	Milk		Feces	
	No.	%	No.	%
P. aeruginosa	14	27.45	17	31.48
P. alkaligenes	12	23.53	14	29.93
P. putida	11	21.57	12	22.22
P. aurefaciens	8	15.69	7	12.96
P. mesophilia	6	11.76	4	7.41
Total	51	100.00	54	100.00

Table 4: serotyping of *Pseudomonas aeruginosa* isolated from the examined milk and fecal samples

Serovars	Milk		Feces	
	No.	%	No.	%
Poly III D	6	42.86	6	35.29
Poly III G	3	21.43	4	23.53
Poly II B	0	0.00	2	11.76
Untypable	5	35.71	5	29.42
Total	14	100.00	17	100.00

Table 5: Characterization of exotoxins extracted from *P. aeruginosa* serovars by using SDS PAGE

Band	Marker		Poly IIIDa		Poly III Db		Poly III Ga		Poly IIIGb		Poly IIBa	
	Mol w.	amount	Mol w.	amount	Mol w.	amount	Mol w.	amount	Mol w.	amount	Mol w.	amount
1	200	17.509	65.117	25.307	60.639	23.449	71.957	23.477	67.569	18.918	64.074	23.153
2	97.40	16.232	60.220	18.918	57.628	6.313	62.202	9.022	60.639	17.281	58.220	20.231
3	68	15.421	50.723	19.629	55.469	6.756	59.114	12.130	54.072	15.902	53	7.599
4	43	16.371	38.967	8.366	52.715	9.411	54.074	17.120	50.220	6.591	50.934	8.065
5	29	16.270	35.897	12.224	48.220	20.163	50.220	7.121	43	5.782	35.120	11.837
6	14.30	18.036	28.717	15.446	43.829	7.756	43	6.634	40.934	18.541	32.001	28.950
7	-	-	-	-	35.313	10.869	38.967	24.351	32.001	16.864	-	-
8	-	-	-	-	24.301	15.133	-	-	-	-	-	-
Sum	-	99.839	-	99.890	-	99.849	-	99.855	-	99.879	-	99.835
In lane	-	100	-	100	-	100	-	100	-	100	-	100

(a) Toxins extracted from feces (b) Toxins extracted from milk

Table 6: Characterization of endotoxins extracted from *P. aeruginosa* serovars by using SDS PAGE

Band	Marker		Poly IIIDa		Poly III Db		Poly III Ga		Poly IIIGb		Poly IIBa	
	Mol w.	amount	Mol w.	amount	Mol w.	amount	Mol w.	amount	Mol w.	amount	Mol w.	amount
1	97.40	21.942	13.244	27.678	12.265	22.853	12.265	19.254	11.456	31.111	12.265	30.367
2	43	22.188	12.265	16.274	11.263	12.772	11.359	17.849	10.520	17.477	10.520	19.153
3	29	17.621	10.520	21.693	10.520	19.912	9.0229	24.097	9.0229	18.941	9.0229	18.964
4	14.30	17.869	9.0229	32.878	7.7390	20.989	6.6377	17.662	7.7390	32.87	7.7390	31.231
5	6.20	20.380	-	-	-	23.217	-	-	-	-	-	-
Sum	-	100	-	98.523	-	99.744	-	98.861	-	99.616	-	99.716
In lane	-	100	-	100	-	100	-	100	-	100	-	100

(a) Toxins extracted from feces (b) Toxins extracted from milk

Table 7: LD $_{50}$ of exotoxin extracted from *P. aeruginosa* serovars

Pseudomonas aeruginosa Exotoxin	Mortality in mice after injection with exotoxins dilutions/µg							LD_{50}/µg
	25	50	75	100	125	150	175	
Poly III D[a]	2	4	6	6	8	9	10	60
Poly III D [b]	3	5	7	7	8	9	10	50
Poly III G [a]	1	2	5	6	6	8	10	25
Poly III G [b]	2	5	6	6	7	8	10	50
Poly II B [a]	0	2	3	5	6	7	9	100

Table 8: LD $_{50}$ of endotoxin extracted from *P. aeruginosa* serovars

Pseudomonas aeruginosa Endotoxins	Mortality in mice after injection with endotoxin dilutions/µg							LD50/µg
	500	1000	1500	2000	2500	3000	3500	
Poly III D[a]	0	3	4	4	4	6	9	2750
Poly III D [b]	1	2	2	3	5	7	10	2500
Poly III G [a]	0	1	3	4	4	6	10	2750
Poly III G [b]	1	3	3	4	5	8	10	2500
Poly II B [a]	2	2	3	4	4	5	8	3000

- The number of inoculated mice in each group (10 mice)
- No deaths were recorded in the control mice group

Table 9: Mortalities in mice inoculated with LD_{50} of exotoxins and endotoxins

Pseudomonas aeruginosa Serotypes	Exotoxins				Endotoxins			
	1st day	2nd day	3rd day	Mortality %	1st day	2nd day	3rd day	Mortality %
Poly III D a	4	1	5	100	10	0	0	100
Poly III D b	3	2	5	100	10	0	0	100
Poly III G a	2	3	5	100	10	0	0	100
Poly III G b	3	3	4	100	10	0	0	100
Poly II B a	0	4	6	100	8	2	0	100

• The number of inoculated mice in each group 10 mices

Table 10: Antibody titre in rabbit injected with exotoxins and endotoxins of different *P. aeruginosa* serotypes

Serovars	Antibody titre	
	Exotoxin	Endotoxins
Poly III D a	1280	640
Poly III D b	640	640
Poly III G a	1280	320
Poly III G b	2560	640
Poly II B a	640	320

Table 11: Passive mice protection of exotoxins and endotoxins rabbit hyperimmune sera after challenge with LD_{50} of exotoxins and endotoxins corresponding to *P. aeruginosa* serotypes

Serovars	Exotoxins antisera		Endotoxins antisera		control
	Challenge with Exotoxins	Challenge with serovar	Challenge with endotoxin	Challenge with serovar	
Poly III D a	10	8	10	2	10
Poly III D b	10	7	9	3	10
Poly III G a	9	7	9	3	10
Poly III G b	10	8	10	3	10
Poly II B a	10	6	10	2	10

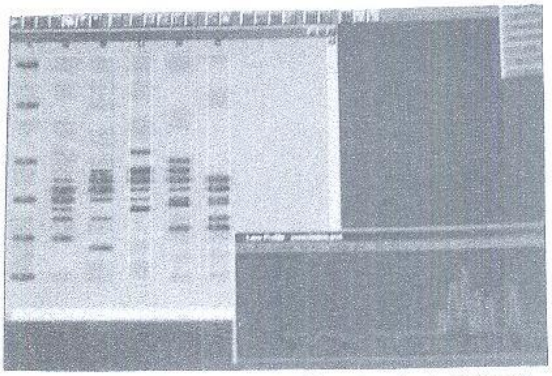

Fig.1 Characterization of exotoxins extracted from P. aeruginosa by using SDS PAGE.

Fig.2 Characterization of endotoxins extracted from P. aeruginosa by using SDS PAGE.