

SDS-PAGE POLYACRYLAMIDE GEL ELECTROPHORESIS IS A RELIABLE METHOD FOR DETECTION OF MASTITIC MILK

Nawar, M. A.

Department of Dairy Science and Technology, Faculty of Agriculture, Alexandria University, Egypt.

ABSTRACT

Individual normal and mastitic milk samples were collected from healthy and udder infected cows. Milk samples were subjected to polyacrylamide gel electrophoretic analysis in order to monitor any considerable changes in milk proteins. At the same time, samples were analysed for the concentration of immune system factors, which increase in milk after infection of the udder. These factors include immunoglobulinG (IgG), lysozyme (LZ) and lactoferrin (LF).

The obtained results revealed that concentrations of IgG, LZ and LF were markedly increased with mastitis incidence and increase in concentrations was mastitis-degree dependent. Since in first degree mastitic milk the concentrations of LZ, LF and IgG were increased by 1.2, 1.5 and 1.3 times, respectively, than in normal milk. But in second and third degrees their concentrations increased by (3.7, 6.7), (4.7, 13.3) and (1.5, 1.8) times for LZ, LF, and IgG, respectively. This result revealed that among the immune proteins, LF and LZ not IgG were highly affected by inflammation of the udder. The electrophoretic patterns of normal and mastitic milk were completely distinguishable. Since the intensities of β - and α s-casein bands were markedly decreased in mastitic milk and the decrease was mastitis degree dependent. On the contrary the intensities of Bovine Serum Albumin (BSA), IgG and LF band were obviously increased in mastitic milk and were positive correlated to degree of mastitis. Meanwhile, some unique peptides were revealed in patterns of second and third degrees of mastitic milk, not present in both

normal and first degree mastitic milk. They have molecular weights of 21.2, 49.8, 54.2, 78.4 and 82.2 kDa. This means that they are related to the intensity of udder inflammation, therefore these peptides can be used as biomarkers for detection of mastitic milk. From these findings it can be concluded that the differentiation not only between normal and mastitic milk but also for detecting the degree of mastitis, can be easily achieved.

Keywords: cow milk, mastitis, antimicrobial factors, immunoglobulinG, lactoferrin, lysozyme, polyacrylamide gel electrophoresis.

INTRODUCTION

Bovine mastitis is one of the most common and also most serious diseases of dairy cows throughout the world and is therefore of considerable economic significance. The prevalence of infectious mastitis among dairy cows is 29% and incidence 62% (Persson, 1992). Most cases of mastitis are caused by bacteria, which usually invade the udder via the teat canal, multiply in the udder and provoke an inflammatory response (DuPreez, 1985). The inflammation of mammary gland is accompanied by major changes in the composition of lacteal secretion especially whey proteins. The changes in whey proteins are thought to be as a result of the destruction of

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permeability barriers, which prevent the passive influx of blood proteins into the alveolar lumen and also to the loss of secretory capability. The combined effect of these two processes would be an increase in concentration of blood serum proteins and decline in udder synthesized proteins. The inflammation of the mammary gland results in increase in immune system factors as lysozyme, lactoferrin and immunoglobulins (Reiter *et al*, 1970; Smith *et al.*, 1971). For detection of mastitis milk, several criteria have been used as indicators of udder inflammation as California Mastitis Test, somatic cell count, level of adenosine triphosphate, N-acetyl-B-D-glucosaminidase activity and serum albumin (Abdurahman *et al*, 1995; Abdurahman, 1996 a&b; Park and Haenlein, 2006).

The present study aimed at finding a relationship between the electrophoretic behaviour of milk proteins in both normal and mastitic conditions on polyacrylamide gel and compare that with the changes in concentrations of immune system factors, i.e., lysozyme, lactoferrin and immunoglobulins in order to establish a proper method for detection of mastitic milk.

MATERIALS AND METHODS

Milk Samples

Cow milk samples were obtained from healthy (20 animals) and infected cows (30 animals) with mastitis (three grades of involution), from the farm of Faculty of Agriculture, Alexandria University.

Chemicals

Bovine lactoferrin, immunoglobulinG and egg white lysozyme (E.C.3.2.1.17) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Lyophilized *Micrococcus lysodieticus* as a substrate for lysozyme was purchased from Difco laboratories, Detroit, Michigan, USA). Low molecular weight standard protein marker was purchased from Bio-Rad, CA, USA.

Methods

Preparation of whey

Whole milk was skimmed by centrifugation at 10000xg for 15 min at 4°C and then pH was adjusted at 4.6 by 1N HCl followed by centrifugation 15 000xg for 20 min at 4°C. The resultant acid whey was neutralized with 1NaOH and filtered using Milipore membrane (0.8 μ m) then kept at -25°C until used in analyses.

Protein determination

Protein content in whey was measured spectrophotometrically according to the method of Bradford (1976).

Concentration of protein samples

Diluted protein samples were concentrated by osmosis through dialysis tubes over solid polyethylene glycol of molecular weight 20,000 according to the method of Hames and Rickwood (1990).

Gel electrophoresis

Analytical slab gel electrophoresis of proteins was conducted in polyacrylamide gel containing 0.1 % sodium dodecyl sulphate (SDS- PAGE) according to the conventional method which involved denaturation of proteins by heating for 5 min in 1% SDS in a boiling water bath prior to applying them

to the gel. The protein concentrations of the samples were adjusted so that approximately 40µg of protein was analyzed. After electrophoresis, proteins were localized in gels using Coomassie blue 0.1 % (Laemmli, 1970).

Protein molecular weight determinations

Molecular weights (MW) of separated proteins on gels were estimated according to the method described by Weber and Osborn (1969) using standard protein markers. After electrophoresis, as well as after gel staining and destaining, both length of the gel and migration distance by the dye were measured. The length of the gel and the positions of the separated proteins were recorded. Mobility of proteins was determined using the following equation:

$$\text{Mobility} = \frac{\text{Distance of protein migration}}{\text{Gel length after destaining}} \times \frac{\text{Length before staining}}{\text{Distance of dye migration}}$$

Mobility was plotted against the known molecular weights expressed on a semi-logarithmic scale.

Immunological Techniques:

Preparation of specific antibodies (antisera)

Specific antibodies to lactoferrin and immunoglobulinG (IgG) for performing radial immunodiffusion assay. Antisera were elicited in rabbits by initial intramuscular injections of 5mg/ml of proteins mixed with Complete Freund's Adjuvant. Booster injections were given intradermally at 3 week intervals. Ten days after last injection, blood was taken from rabbits by heart puncture and serum was prepared, then antiserum's titer was determined (Johnstone and Thorpe, 1985).

Radial immunodiffusion assay

For determination of lactoferrin and IgG the radial immunodiffusion technique was used (Mancini *et al*, 1965). Glass slides were covered with agarose (1.5%) in 0.05 M barbital buffer, pH 8.2 mixed with the specific antiserum (to lactoferrin or IgG). Wells of 4mm in diameter were made then 10 µl of whey were applied in each well. The diameter of the precipitation circle around wells were measured. A standard curve was constructed by plotting the log of the lactoferrin or IgG concentration against the diameter of the precipitation circle.

Lysozyme activity assay

The lysoplate assay method was used as described by Lie *et al*. (1986). Glass slides were covered with agarose 1% mixed with *Micrococcus lysodieticus* in 0.05 M phosphate buffer, pH 7.4. Wells of 4mm in diameter were made. Each well received 15 µl of whey or standard lysozyme. Slides were left for 30 min for diffusion at room temperature then incubated for 18 h at 37°C. The zone of lysis around wells were measured. A standard curve was constructed by plotting the log of the lysozyme concentration against the diameter of the lysis zone.

RESULTS AND DISCUSSION

The first line of defence against infection in the bovine udder is the teat canal. If bacteria pass this barrier and enter the teat cistern they meet the second line of defence- cellular i.e. leukocytes and humoral immune system

factors. Immunoglobulins, lysozyme and lactoferrin are the components of the humoral immune system. Humoral factors work together with cellular defence in milk (Outteridge and Lee, 1988). Therefore in order to monitor the changes in milk composition due to udder infection, the humoral immune system factors concentrations in normal and mastitic milk were determined.

Table (1) and Fig. (1) show the concentrations of lysozyme(LZ), lactoferrin (LF) and immunoglobulinG (IgG) in normal and mastitic milk. It was clear that concentrations of all of these proteins were markedly increased with mastitis incidence. It was noticed also that their increases in concentrations were mastitis-degree dependent. Since in first degree mastitic milk the concentrations of LZ, LF and IgG were increased by 1.2, 1.5 and 1.3, respectively, than normal milk. But in second and third degrees their concentrations increased by (3.7, 6.7), (4.7, 13.3) and (1.5, 1.8) for LZ, LF, and IgG, respectively. This result revealed that among the immune proteins, LF and LZ not IgG were highly affected by inflammation of the udder. This result is in agreement with the literature (Harmon and Newbould, 1980; Carlsson *et al*, 1989) especially for LF. Therefore it can be concluded that the concentration of either LF or LZ in milk can be considered a biomarker for the detection of mastitic milk. Meanwhile the results showed that the changes in IgG concentration was moderate comparing to that of LZ or LF. Other researchers found that immunoglobulins are low in normal milk but increase during mastitis in lactated cows (Guidry *et al*, 1983; Andersson *et al*, 1986). Reiter (1985) reported that normal milk contains trace amounts of LZ but higher levels are present in mastitic milk.

Figures 2 & 3 show the electrophoretic patterns of normal and mastitic cow milk. The patterns revealed the appearance of several peptide bands differed in migration positions, band intensity, numbers and molecular weights. The major peptide bands referred to casein fractions (β and α -caseins), while minor peptide bands referred to whey proteins (serum albumin (SA), lactoferrin (LF) and immunoglobulin G (IgG)). It was clear that the pattern of normal milk is extremely different than that of mastitic milk, since the concentrations of both major and minor proteins were different (Table2). β - and α _S-casein concentrations were decreased in mastitic milk samples. At the same time their decreases were pronounced in milk samples of second and third degrees of mastitis. This result revealed that the synthesis of casein fractions were decreased with udder infection and it was highly pronounced in acute degree of inflammation. Therefore it can be concluded that the changes in electrophoretic patterns of β - and α _S-casein fractions in bovine milk can be used a biomarker for detection of mastitic milk. Lohuis *et al*. (1988) stated that in bovine milk the mammary secretions undergo many compositional changes during inflammation of the udder. The synthesis of lactose, casein and fat decreases, however, leading to reduced milk production.

Concerning the changes in whey proteins in normal and mastitic milk, the electrophoretic patterns (Fig. 3) showed the appearance of several peptide bands in all whey samples. These bands were differed in migration, number, intensities and molecular weights. For instance, in normal milk whey, 6 peptide bands were separated on the gel versus 7, 10 and 11 bands for whey samples represent first, second and third degree of mastitis. Meanwhile

the major bands in all patterns were IgG, LF and BSA. The concentrations of these proteins not only differed markedly between normal and mastitic milk but also among the three types of mastitic milk (Table 3). Their concentrations were increased with the increase of inflammation of the udder. The patterns showed also that in second and third degree mastitic milk, the intensity of LF, IgG and BSA bands were markedly increased. Lohuis *et al.* (1988) found that inflammation of bovine udder resulted in increase in serum proteins like albumin, antitrypsin and immunoglobulins. Likewise, the concentrations of the proteolytic enzyme (Plasmin), the intracellular enzyme (NAGase), antibacterial proteins (lysozyme and lactoferrin), several hydrolases and alkaline phosphatase also increase.

Table (1): Concentration of antimicrobial factors in normal and mastitic cow milk (Means \pm SEM)

		LZ ($\mu\text{g/ml}$)	IgG (mg/ml)	LF (mg/ml)
		$\bar{X} \pm \text{SEM}$		
Normal milk		0.198 \pm 0.063	0.715 \pm 0.441	0.150 \pm 0.061
Mastitis grades	1 st	0.230 \pm 0.067	0.915 \pm 0.434	0.225 \pm 0.105
	2 nd	0.725 \pm 0.027	1.035 \pm 0.249	0.703 \pm 0.345
	3 rd	1.330 \pm 0.854	1.275 \pm 0.357	1.990 \pm 0.953

LZ : Lysozyme.

IgG : Immunoglobulin G.

LF : Lactoferrin

\bar{X} : Mean

SEM: Standard error of mean.

Table (2): Total lab analysis of Fig. 2 of SDS-PAGE of normal and mastitic milk

Band No	Position	Rm	Mw (kDa)	Normal	1 st	2 nd	3 rd
1	23	0.028	117.0	4.35	3.06	5.36	6.21
2	84	0.054	99.8	9.84	10.64	10.89	11.43
3	123	0.152	97.0	1.02	0.98	1.93	0.86
4	172	0.213	87.8	1.91	0.88	1.64	0.59
5	274	0.339	79.9	0.78	1.73	1.68	0.58
6	324	0.401	76.7	0.84	2.94	4.35	1.82
7	344	0.425	72.0	1.21	1.32	1.00	1.39
8	404	0.499	66.2	5.21	4.39	18.83	17.19
9	425	0.525	25.1	31.97	30.90	20.25	24.31
10	613	0.834	23.2	27.50	25.90	14.60	15.36
11	675	0.921	22.0	8.87	7.80	15.10	15.90
12	744	0.977	20.5	5.20	5.41	2.55	2.50
13	790	0.998	18.2	1.30	1.96	1.82	1.86

R.m: Relative mobility.

Mw (kDa): Molecular weight in kilo Dalton.

1st, 2nd and 3rd: Degrees of mastitis.

The electrophoretic patterns revealed also the presence of some unique peptide bands in whey samples of third degree mastitic milk. They have molecular weights of 82.2 and 78.4 kDa. In addition to that another two peptide bands (54.2 & 49.8 kDa) appeared in both second and third degree mastitic milk samples. At the same time a unique peptide band (21.2 kDa)

was only appeared in the second degree mastitic milk. The appearance of such peptides in mastitic milk of high degrees of inflammation may be considered an expression of the defence system of the udder against infection by synthesis of such proteins.

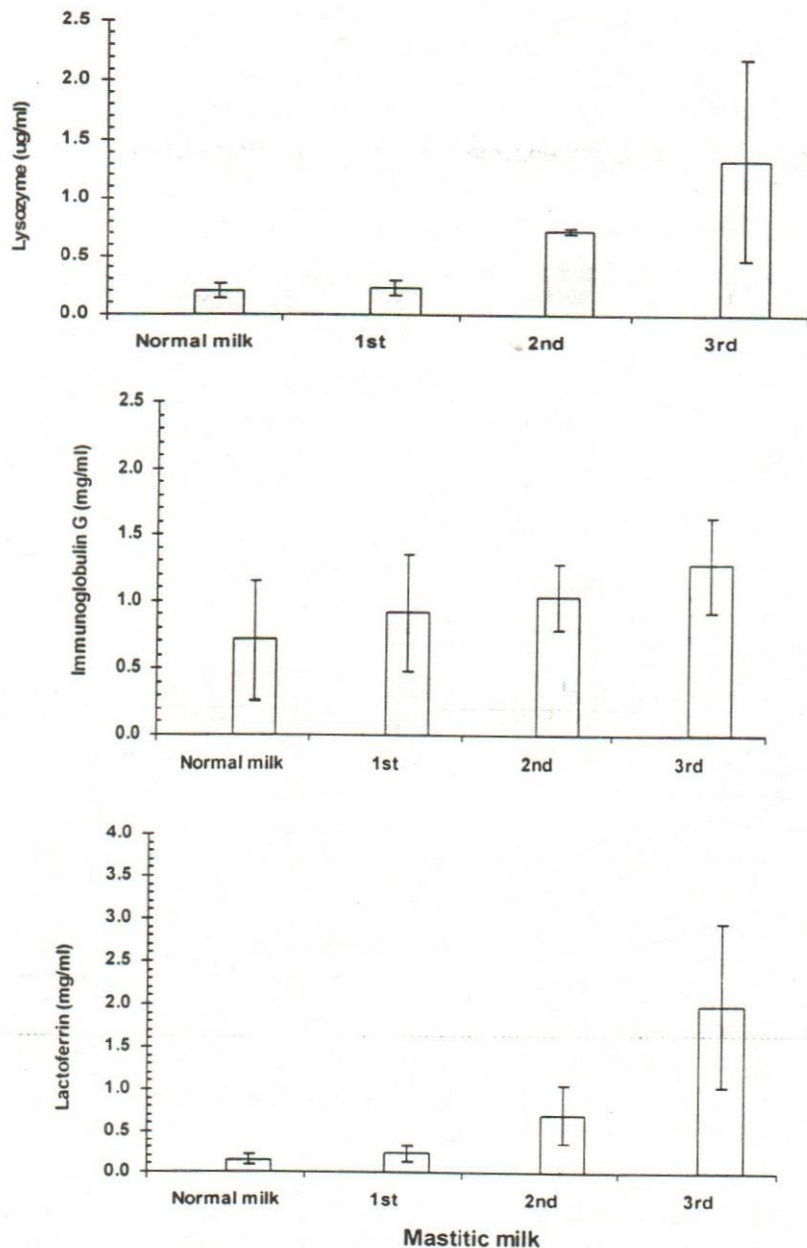


Fig. (1): Concentrations of antimicrobial factors in normal and mastitic cow milk (Means±SEM: Standard error of mean)

From these results, it can be concluded that the distinguished electrophoretic patterns of casein fractions and whey proteins especially BSA, IgG and LF can be considered a reliable way for easily detection of mastitic milk but also for the differentiation among the various cases of mastitis.

Table (3): Total lab analysis of Fig. 3 of SDS-PAGE of whey of normal and mastitic milk

Band No	Position	Rm	Mw (kDa)	Normal	1 st	2 nd	3 rd
1	12	0.015	104.0	3.85	3.65	1.24	1.19
2	29	0.037	99.8	22.09	24.24	20.54	14.03
3	72	0.092	93.2	12.30	3.10	4.64	2.72
4	96	0.125	82.2	---	---	---	2.02
5	102	0.134	78.4	---	---	---	2.00
6	115	0.157	76.7	0.90	1.23	1.40	1.71
7	151	0.192	66.2	58.02	62.68	64.42	70.60
8	187	0.236	54.2	---	---	0.96	1.60
9	193	0.242	49.8	---	---	0.98	1.22
10	205	0.261	41.1	2.84	3.00	1.16	0.72
11	226	0.288	23.4	---	2.10	2.20	2.19
12	249	0.316	21.2	---	---	2.45	---

Rm: Relative mobility.

1st, 2nd and 3rd: Degrees of mastitis.

Mw (kDa): Molecular weight in kilo Dalton.

---: Not present

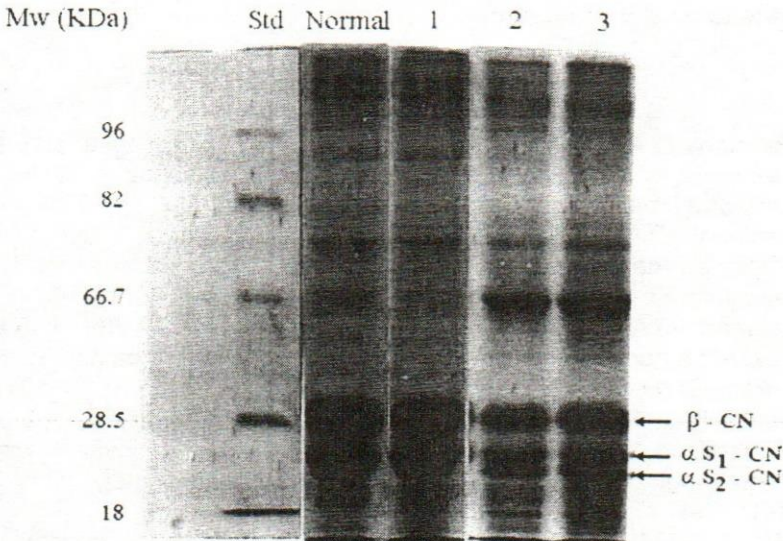


Fig. (2): SDS-PAGE (10%T) of normal and mastitic cows' milk. 1, 2 & 3: Mastitic milk samples representing 1st, 2nd and third degree of mastitis, respectively. Std: Standard protein marker, β -CN, α_{S1} - & α_{S2} -CN: \square -casein, \square_{S1} - & \square_{S2} -casein.

Anode is toward bottom of photo

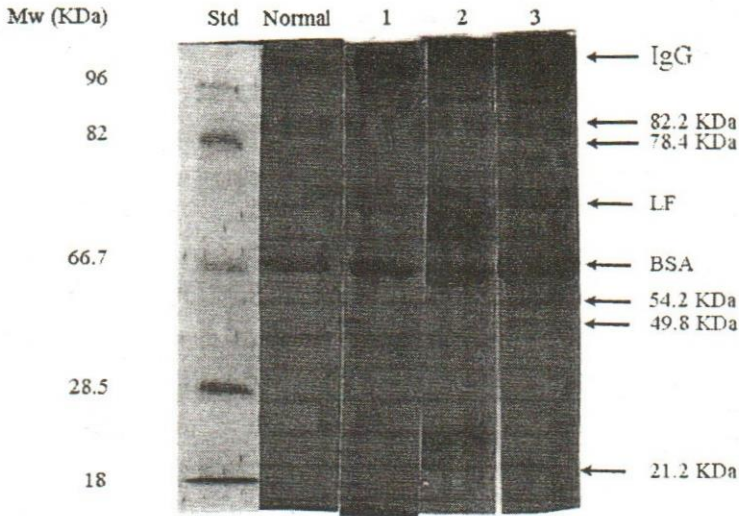


Fig. (3): SDS-PAGE (10%T) of normal and mastitic cows' milk whey samples. 1, 2 & 3: Mastitic milk whey samples representing 1st, 2nd and third degree of mastitis, respectively. Std: Standard protein marker, IgG: immunoglobulin G; LF: lactoferrin; BSA: bovine serum albumin.

Anode is toward bottom of photo.

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طريقة الفصل الكهربى باستخدام جل البولى اكريلاميد فى وجود الـ SDS تعتبر
طريقة مقبولة للكشف عن الالبان الناتجة من الحيوانات المصابة بمرض حمى
الضرع
محسن نوار
قسم علوم وتكنولوجيا الالبان - كلية الزراعة - جامعة الإسكندرية

الغرض من هذا البحث هو تقييم طريقة من طرق التحليل المتقدم لبروتينات اللبن لمعرفة التغيرات
الحادثة بها وإمكانية تقييم ذلك لمعرفة مدى إمكانية استخدامها كطريقة من طرق التعرف على الالبان الناتجة
من حيوانات مصابة ولذا تم تجميع عينات فردية من ألبان أبقار مصابة بحمى الضرع بدرجات مختلفة
(بسيطة، متوسطة، قوية) وكذلك عينات من أبقار غير مصابة وتم تحليل تلك العينات بطريقة الفصل
الكهربى على جل الـ SDS وتم كذلك تقدير تركيزات البروتينات التى تشكل النظام
المناعى فى اللبن وهى انزيم الليوزيم، اللاكتوفيرين و بروتين المناعة G، وقد اوضحت النتائج ان تركيز
جميع بروتينات النظام المناعى ازدادت فى البان الحيوانات المصابة وان الزيادة كانت ذات علاقة طردية
مع شدة الإصابة بالمرض حيث بلغت الزيادة فى تركيزات الليوزيم، اللاكتوفيرين، بروتين المناعة G
(١,٢، ١,٥، ١,٣، ٣,٧، ٤,٧، ١,٥)، (٦,٧، ١٣,٣، ١,٨) فى ألبان الأبقار المصابة بالدرجات الأولى
والثانية والثالثة من المرض على الترتيب.

وقد اوضحت انماط الهجرة الكهربية لبروتينات الالبان الناتجة من ابقار مصابة وسليمة انه يوجد
اختلاف واضح تماما بين النوعين من حيث عدد البروتينات المنفصلة وكذلك تركيز تلك البروتينات ومواقع
هجرتها على مادة الجل. وقد انخفض تركيز كل من البيتا كازين والالفا اس كازين انخفاضاً ملحوظاً فى
انماط البان الحيوانات المصابة، وعلى النقيض فان تركيزات كل من البيومين السيرم، وبروتين المناعة G،
واللاكتوفيرين قد ارتفعت فى البان الأبقار المصابة، وكانت الزيادة مطردة مع شدة الإصابة. كذلك اظهرت
انماط الهجرة انه مع زيادة شدة الإصابة بالمرض ظهرت بعض الببتيدات ذات اوزان جزيئية ٢١,٢، ٤٩,٨،
٥٤,٢، ٧٨,٤، ٨٢,٢ كيلو دالتون مما يعنى ان تلك الببتيدات ذات علاقة بالمرض وبالتالى يمكن
الاعتماد عليها وعلى التغيرات الواضحة فى سلوك البروتينات الأخرى كأدلة حيوية للكشف عن الإصابة
بمرض حمى الضرع وكذلك تحديد درجة الإصابة.

وبناء على تلك النتائج المتحصل عليها يمكن استنتاج ان طريقة الفصل الكهربى يمكن اعتبارها
طريقة مرجعية للكشف عن البان الأبقار المصابة بمرض حمى الضرع وكذلك لتحديد درجة الإصابة.