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The Role of Anaerobic Bacteria in the Dairy Cattle Mastitis

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SUMMARY

One hundred and ten milk samples were collected from 35 dairy cows with recurrent mastitis and 75 apparently healthy ones. The field application of California Mastitis Test (CMT) on the apparently healthy cows indicated 60% (45 out of 75 cows) with sub-clinical mastitis. The estimation of SCC recorded 31.8% (35 samples) above $1x10^6$ cell/ml, 40.9% (45 samples) were ranged between $4x10^5$ to $1x10^6$ cell/ml and 27.3% (30 samples) were less than $4x10^5$cell/ml. The bacteriological examination revealed 10% facultative anaerobic bacteria from normal milk samples, while both facultative and obligatory anaerobic bacteria were 62.2% from sub-clinical and 100% from clinical mastitic milk samples. The highly effective antibiotics on the isolated bacteria in the laboratory were florofenicol, tetradelta, gentamicin, amoxicillin-clavulanate, refampicin and metronidazol.

INTRODUCTION

Mastitis, an inflammatory reaction of the mammary glands, is usually caused by microbial infections and is recognized as the most costly disease in dairy cattle (Zhao and Lacasse, 2006). The anaerobic bacteria play an important role in dairy cattle mastitis, this role was previously investigated (Preez *et al.*, 1981). Routine bacteriological diagnosis of bovine mastitis does not provide an index to the obligate anaerobic flora involved. No anaerobic bacteria were recovered from cows with normal quarters or those with latent facultative anaerobic or aerobic udder infection as diagnosed according to the Criteria of the International Dairy federation Preez and Greef (1984). Anaerobic bacteria have been isolated from lactating as well as dry cows. Most anaerobic bacteria were isolated concurrently with facultative anaerobic bacteria except in aseptic mastitic cases. The polymicrobial nature of the udder infections show that multiple anaerobic as

well as facultative anaerobic species colonies are act together (Preez 1989). No nationwide studies of the incidence rate of clinical mastitis (IRCM) have been conducted, because IRCM and distribution of mastitis causing bacteria may show substantional geographic variation, Specific IRCM. (Olde Riekerink *et al.*, 2008). It is stressed that the udders of all heifers should be examined daily so that cases of mastitis can be treated immediately (Jonsson *et al.*, 1991). The aim of this study was: diagnosis of anaerobic mastitis in the dairy farms. In addition to rapid conducting of proper treatment and control measures of anaerobic mastitis in the dairy farms.

MATERIAL AND METHODS

Sample collection:-

Milk samples from 110 cows were collected from private dairy farms. Initially each cow was examined clinically; particular attention was given to the condition of the under. 75 animals were clinically healthy and showed no mammary disorder, while 35 animals had recurrent mastitic udders. Each teat was disinfected with cotton soaked in 70% ethyl alcohol. Sampling was carried out after morning milking, 10-20 ml of milk were squirted into sterile universal containers. The samples were transported to the laboratory ice cooled. The samples were analysed using standard methods described by Hogan *et al.* (1999) and Quinn *et al.* (2000).

California Mastitis Test (CMT):-

CMT was applied as a cow-side test after quarter-milk sampling. The results were read and evaluated according to the manufacture' s instructions (Schalm *et al.*, 1971). Scores represented four categories: 0, negative; 1 trace; 2, weak positive; 3, distinct positive; 4, strong positive.

Somatic cell counters (SCC):-

SCC in all milk samples was estimated by Soma count 150 (BENTLY co.; Germany).

Bacteriological examination:-

Standard procedures were used for isolation and identification of anaerobic bacteria from all collected samples (110) as previously described by (Koneman *et al.*, 1983 and Quinn *et al.*, 2002). The growing suspected colonies were described for their appearance, haemolytic activity and morphological characters. A smear from colony was stained with Gram's stain and examined microscopically from each plate. The identification of the isolated anaerobes, either facultative or obligatory anaerobic bacteria was conducted by streaking of

colonies on manitol salt agar for *staphylococcal* isolates (Merlino *et al.*, 1996) and cultivation on blood agar plates for determination of *peptococci*, *E.coli*, *B.fragilis* and Clostridia (Tutenel *et al.*, 2003). Clostridium species mainly *C. perfringens* were incubated anaerobically at 37 C for 24 hours. The growing colonies were tested by catalase test (Konemann *et al.*, 1983 and Cruickshank *et al.*, 1996). The negative catalase colonies were purified by streaking a loopfull onto C. *perfringens* reinforced agar plates and incubated anaerobically at 37 C for 24 hours. The growing colonies were identified by morphological and biochemical characters (Chai, *et al.*, 2007). The anaerobic bacteria of the inoculated fresh milk sample were inoculated into cooked meat broth (Oxoid) and streaked onto sheep blood agar plates 10% (Oxoid) with 70 ug /ml neomycin sulphate after 24 hours with anaerobic incubation at 37C.

The determination of the toxigenic activity and typing of the isolated *C. perfringens*:-

This was done by using the pathogenisty test in Guinea pigs Willis, (1964) and mice neutralization test (Smith and Holdman; 1968 and Itodo, 1991). The typing of isolated colonies by the using of the Enzyme Linked Immuno-Sorbent Assay (ELISA) using antisera obtained from (Abasia) Vaccines and Sera Veterinary Research Institute (El-Idrissi and Ward, 1992) with some laboratory modifications to facilitate the detection and estimation of the antitoxin titers against *C. perfringens* types "A", "B" and "D" toxins in the separated whey collected from the milk samples of the dairy cows according to Krt, (1999) and Uzal *et al.* (2003). Briefly, Microtitire plates were coated (100 µl/well) each with washed whole cell antigen dilution of Alpha, Beta and Epsilon toxins using saline 1:50. The coated plates were incubated over night and washed. The blocking step using bovine serum albumen, then washing, and inoculating each well with 50 µl then incubation at 37 C for 30 min. Then adding antibody bovine conjugate 100µl in each well then washing three times then adding the OPD substrate diluted by methanol and H_2O_2. After I hour washing 3 times then stopping the reaction using 1:10 H_2SO_4 by adding 100 µl to each well and reading by ELISA reader after 10 min. according to Byrne *et at.* (2000).

Sensitivity test:-

For choosing the suitable antibiotics in the treatment of the clinically mastitic cows. The sensitivity test was carried out using Mueller-Hinton blood agar (Garctikyaa-Rodriguez *et al.*, 2005). Amoxicillin, penicillin, ampicilline, chlorophenicol,

rifampicine, metronidazol, gentamicin, erythromycin, neomycin, doxycycline, florophenicol, amoxicillin-clavulanate, tetradelta and tetracycline disks were used. The isolates were categorized as susceptible (sensitive), intermediate or resistant according to the methods and criteria described by National Committee for Clinical Laboratory Standards (NCCLS, 2002).

RESULTS

Results of estimation of SCC of the fresh milk samples by the using of the CMT and the milk scan apparatus:

As shown in Table 1, it was clear that 45 out of the 75 apparently normal samples (60%) had SCC counts between $4x10^5$ - $1x10^6$ indicating cases of subclinical mastitis. The remaining 30 samples had counts less than $4x10^5$, i.e. a score between 0 and 1 which means normal milk.

The results of bacteriological examination:-

Result of the identification and typing of facultative and obligatory anaerobic bacteria:

As illustrated in Table 2, the percentages of isolated facultative and obligatory anaerobic bacteria were 35.5% and 24.5%, respectively.

As demonstrated in the Table 3, the different isolated anaerobic bacteria from dairy cattle mastitis included *E. coli* represented by 22.7% and *staphylococci* (either coagulase +ve or –ve were 8.2% and 4.6% respectively). *C. perfringens*, *B. fragilis* and *Peptococcus* species were 7.3%, 10.9% and 6.4%, respectively. The obligatory anaerobic Gm +ve bacilli were determined as *C. perfringens* types A, B, C and D.

The determination and typing of the *C. perfringens*:

Typing was determined by ELISA. The isolated *C. perfringens* types represented by 7.2%. The results of the determination of the antitoxin titers in the milk samples of the affected dairy cows by *C. perfringens* types were tabulated in (Table 4), which reported that the incidence rates of *C. perfringens* types were (29.6%) type A, (7.4%) type C, (11.1%) type D and (11.1%) type B, respectively.

The results of Sensitivity test:

The highly effective antibiotics on the isolated bacteria in the laboratory were florofenicol, tetradelta, gentamicin, amoxicillin-clavulanate, rifampicin and metronidazol.

Table 1: Estimation of SCC of the fresh 110 milk samples

The state of the udder	The No. of examined samples	The scores	Gelling formation	Average of SCC	The percentages	Interpre – tation
Apparently normal udder	10	0	None	$< 4x10^5$	27.3%	Normal udder
	20	1	Trace			
	45	2	Slight	$4x10^5$ to $1x10^6$	40.9%	Subclinical mastitis
Recurrent mastitic udder	18	3	Moderate	$>1x10^6$	31.8%	Clinical mastitis
	17	4	Heavy			

Table 2: Incidence of the facultative and the obligatory anaerobic bacteria

The state of the udder	The No. of examined samples	The isolated facultative anaerobic bacteria	The No. of isolated bacteria and (%)		The isolated obligatory anaerobic bacteria	The No. of isolated bacteria and (%)	
Apparently normal udder	30	*E. coli*	3	(10%)	---------	-------	
Subclinical mastitic udder	45	*E. coli* *Staphylococci*	10 6	(35.6%)	*C. perfringens* *B. fragilis* *Peptococci*	3 5 4	(26.7%)
Recurrent mastitic udder	35	*E. coli* *Staphylococci*	12 8	(57.1%)	*C. perfringens* *B. fragilis* *Peptococci*	5 7 3	(42.9%)
Total	110	39		35.5%	27		24.6%

Table 3: Identification of isolated Bacteria

Bacteria	No.	Percentages
E. coli	25	22.7%
Staphylococci	14	12.7%
C. perfringens	8	7.3%
B. fragilis	12	10.1%
Peptococci	7	6.4%

Table 4: Determination and typing of *C. perfringens*

Antitoxins of *C. perfringens*			
Alpha (%)	Beta (%)	Epsilon (%)	Beta and Epsilon (%)
8 (29.6%)	2 (7.4%)	3 (11.1%)	3 (11.1%)

DISCUSSION

Anaerobic mastitis is very important condition in the dairy farms, causing reduction in milk production, damage of the udder tissue as well as complete losing of the animal. In this work, 110 milk samples were collected from lactating cows for detection of anaerobic mastitis (Gangrenous mastitis).

CMT was used for indirect measuring of SCC (Perry *et al.*, 1987). This test is considered a sharpe line between the different degree of subclinical mastitis and early detection of clinical mastitis (El-Rashidy *et al.*, 1986 and Rosenberg *et al.*, 2002). The CMT measures the 4 quarters at the same time thus helping in the treatment or culling. The subclinical cases could be treated by either stripping after milking the cow or more milking e.g. 4 or 6 times /day. Application of CMT on the apparently normal cow's udder indicated that 45cows (60%) had subclinical mastitis. The subclinical mastitis leading to loss in milk production nearly to three times of clinical mastitis (Seddek *et al.*, 1999).This percentage of subclinical mastitis is high and confirms the hypothesis of National Mastitis Council, (1997), which reported that each case of clinical mastitis associated with 15-20 cases of subclinical mastitis in dairy farms.

Similar result 43.6% was illustrated by (Chanders and Baxi, 1975 while higher incidence 60% was reported by (Abdel-Karim and El-Ashmawy, 1979). The incidence of clinical mastitis was (31.8%), which was high due to improper diagnosis and treatment of the clinical cases of mastitis that were complicated with anaerobic bacteria leading to severe cases of recurrent (gangrenous) mastitis (Fagliari *et al.*, 1989), who diagnosed clinical mastitis in 50 quarters of 50 cows, when the survey was done on dairy farms, where the standard of hygiene was quiet good (Mc Dougall, 1999). There is a good correlation between the number of SCC and the clinical finding of the udder (Viana *et al.*, 1987). The SCC less than $4x10^5$ indicated normal milk and apparently normal udder, while from $4x10^5$ to $1x10^6$ indicated subclinical mastitis but the result above $1x10^6$ indicated the

mastitic udder.

Mastitis is a multifactorial complex disease that can be induced by different pathogenic bacteria, which may be categorized under contagious, environmental and miscellaneous causes Schalm and Woods (1953). The bacteriological studies on the collected milk samples (110) in the present study indicated that the isolated bacteria, either facultative (35.5%) or obligatory (24.5%) anaerobic bacteria, represented about (60%) i.e. 66 out of 110.

As shown in Table 2, it is noted that no obligatory anaerobic bacteria were isolated from milk obtained from normal udder. This result is in agreement with the statement of Preez and Greef (1984), who reported that no anaerobic bacteria were recovered from cows with normal quarters as diagnosed according to the Criteria of the International Dairy federation.

On the other hand, both facultative and obligatory anaerobic bacteria were recovered from cows of subclinical and clinical mastitis. These were stressed already by Preez (1989) and indicate the importance of including the detection of obligatory anaerobic bacteria in the routine diagnosis of mastitis. The present study illustrated the role of obligatory anaerobic bacteria, particularly in cases of recurrent mastitic udder, when the incidence of such bacteria was much higher than in cases with subclinical mastitis. This finding confirms with that reported by other authors (El-Rashidy *et al.*, 1986 and Chai, *et al.*, 2007).

The results of ELISA in typing of *C. perfringens* showed that various toxigenic types were involved in cases of mastitis. This was already mentioned by (Hamouda *et al*, 2009).

The highly effective antibiotics on the isolated bacteria in the laboratory as a result of sensitivity test (Jousimies-Somer *et al.*, 1996) were florofenicol, tetradelta, gentamicin, rifampicin and metronidazol. The use of these antibiotics in the field did not provide the highly effectiveness probably because some isolated bacteria (*B. fragillis*) secrete beta-lactimease enzyme which destroys the beta lactame ring in some antibiotics (Preez *et al.*, 1985), and other bacteria secrete the highly toxic substances (exotoxins) that induce the disease condition (*C. perfringens)*. In these cases the use of antibiotics was useless as they did not affect the toxins causing the disease condition. The control measures are very important especially the application of the proper regime of vaccination for elevation of the immune status of the udder against the invader bacteria.

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دور الميكروبات اللاهوائية فى التهاب الضرع فى الأبقار الحلابة

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معهد بحوث التناسليات الحيوانية بالهرم

هذه الدراسة تمثلت فى تجميع عدد ١١٠ عينة لبن من الأبقار الحلابة منهم ٣٥ بقرة حلوب مصابة بالتهاب ضرع متكرر و ٧٥ بقرة حلوب سليمة ظاهريا.وقد تم اجراء اختبار الكليفورنيا لالتهاب الضرع فى المزرعةعلى الابقار السليمة ظاهريا وكانت النتيجة ٦٠%(٤٥ : ٧٥) مصابه بالتهاب الضرع الغير ظاهرى. تم تحديد عدد الخلايا الجسيمية وكانت نتيجتها ٣٠,٨% أكثر من ١x١٠^٦خلية /ملليلتر و ٤٠,٤%(٤٥عينة) تتراوح عددها بين ٤X١٠^٥ الى ١٠^٦/خلية/ملليلتر. و٢٧,٣%(٣٠ عينة) كان العدد للخلايا الجسيمية اقل من ٤x١٠^٥خلية /ملليلتر. البكتيريا اللاهوائية الاختيارية كانت فى هذة الدراسة نسبتها ١٠% من عينات اللبن من الابقار السليمة ظاهريا. بينما كل من البكتيريا الاختيارية والاجبارية النمو فى ظروف اللاهوائية كانت نسبتها معا ٢٦,٢% من عينات اللبن من الأبقار المصابة بالتهاب الضرع الغير ظاهرى. بينما كانت نسبتها ١٠٠% فى عينات اللن من الأبقار المصابة بالتهاب الضرع الظاهرى. الفلوروفينيكول و التيترادلتا و الجنتامايسين والريفامبيسين والمترونيدازول وجدوا أنهم الأكثر فاعلية فى التاثير على البكتيريا المعزولة ولكنها كانت غير مؤثرة فى الحقل قى المزارع بنفس الكفاءة فى المعمل ولهذا يجب أن نعمل على رفع الكفاءة المناعية لنسيج الضرع وهذة هى أحسن وسيلة للسيطرة على الاصابة البكتيرية اللاهوائية للضرع.