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BACTERIOLOGICAL STUDIES ON PATHOGENS CAUSING SUB-CLINICAL MASTITIS IN HOLSTEIN-FRIESIAN DAIRY COWS IN ASSIUT GOVERNORATE

(With 3 Tables)

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دراسات بكتريولوجية على المسببات البكتيرية لالتهاب الضرع الخفي في أبقار الهولشتين الفريزيان الحلابة بمحافظة أسيوط

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أجريت الدراسة على أبقار الهولشتين فريزيان الحلابة والتي تعاني من تكرار الإصابة بالتهاب الضرع الخفي. وبفحص عدد ١١٠عينة لبن مجمعة من الأرباع للبقرة الواحدة حقليا باختباري الكاليفورنيا ماستيتس والهويتسيد المعدل. أسفرت النتائج عن ايجابية الفحص في عدد ٣٥و٣٧ عينة منها لكلا الاختبارين على التوالي أصابتها بالتهاب الضرع الخفي. وقد خضعت هذه العينات الإيجابية للاختبارات الحقلية للفحص البكتريولوجي وتم زرعها على المستنبتات العامة والخاصة لزيادة فرص العزل حيث عزلت عدد ١٠٣عترة تمثل مجموعتين من المسببات البكتيرية: عالية الضراوة ٣٧عترة بنسبة ٣٥,٩٢%وميكروبات التلوث البيئي ٦٦عترة بنسبة ٦٤,٠٨%. وقد تم عزل أكثر من عتره من الميكروبات الممرضة في العينة الواحدة من ٣٤عينة لبن بنسبة ٩٧,١٤% وكانت أغلبها تحتوى على ثلاث عترات مختلفة في العينة الواحدة بنسبة٥٧,١٤%. تمثلت ميكروبات عالية الضراوة في عزل المكور العنقودى الذهبى ١٦,٥% والسبحي أجلاكتيا ١٠,٦٨% والكورينى باكتيريم ٧,٧٧% والسبحى ديسجلاكتيا ٠,٩٧%. أما بالنسبة لميكروبات التلوث البيئي فقد تمثلت في إنتيروباكتيرايروجينز ١٢,٦٢% وكل من الميكروب القولونى والإنتيروكوكاس فيكالس ١٠,٦٨% لكل منها والسبحي إكواى ٦,٨% والمكور العنقودى السابروفيتكس٤,٨٥ % وعترات أخرى بنسب أقل كان من أبرزها الميكروب القولوني (أو-١٥٧) بنسبة ١,٩٤%. وبإجراء اختبار الحساسية لكل من هذه العترات على حدة ضد ١٠من المضادات الحيوية المختلفة أسفرت النتائج عن حساسية جميع العترات المعزولة للسيبروفلوكساسين والجنتاميسين بنسبة ١٠٠% و ٨٠,٨٤% علي الترتيب. ونوقشت النتائج على ضوء ما تقدم.

SUMMERY

The study was conducted on 110 Holstein-Friesian dairy cows suffering from recurrent sub-clinical mastitis. Screening of 110 milk samples, pooled samples, by using of both field tests (California Mastitis Test and

modified Whiteside Test), reveled that 35 and 37 milk samples showed positive by both tests, respectively. These positive samples were examined bacteriologically on general and specific enriched media. The isolated bacterial strains (103 isolates) resembled two categories: contagious bacteria 37 isolates (35.92%) and environmental bacteria 66 isolates (64.08%). Thirty four (97.14%) milk samples showed mixed infection, where most of them 20 milk samples (57.14%) were infected with triple infection. The isolated contagious strains were *Staph. aureus* 17 (16.5%), *Strept. agalactia* 11 (10.68%), *Corynebaterium* spp. 8 (7.77%) and *Strept. dysgalactia* 1 (0.97%), while the environmental bacteria were *Enterobacter aerogenes* 13 (12.62%), *Enterococcus faecalis* and *E. coli* 11 (10.68%) for both, *Strept. equi subsp. zooepidemicus* 7 (6.8%), *Staph. saprophyticus* 5 (4.85%) and other strains with less proportions were isolated where the most highly pathogenic of them was *E. coli* O_{157} (1.94%). Antimicrobial susceptibility testing reveled that all isolated strains were sensitive to ciprofloxacin and gentamycin with percentage 100% and 80.84%, respectively.

Key words: *Sub-clinical mastitis, bacteriological examination, Holstein-Friesian cows*

INTRODUCTION

Mastitis is the most frequent and prevalent production disease in dairy herds. It is a widely health problem does not only cause the largest economic disease related losses in dairy herd farm; but it is also responsible for extended usage of antibiotic in these enterprises (Varshney and Naresh, 2004; Bannerman, *et al.*, 2008 and Sakai, *et al.*, 2008). The serious effect created by mastitis are mostly due to its subclinical form (APHA, 1985).

The majority of udder infections are caused by pathogens of two categories, including contagious bacteria, that spread from an infected cow to another one, such as: *Staphylococcus aureus, Streptococcus agalactiae,* and *Corynebacterium bovis* (Harmon and Langlois, 1986; National Mastitis Council, 1987, Simth and Hogan,1995; El-Balkemy, *et al.*, 1997; El-Khodery and Hoedemaker, 2005 and Abdel-Khalek and El-Sherbini, 2005). *Streptococcus* dysgalactiae have some characteristics of contagious organism and spread from cow to another (Smith and Hogan, 1995 and El-Balkemy, *et al.*, 1997). The other category environmental bacteria that are commonly present in surrounding environment and may reach the teat end from that source, such as: *E. coli*, *Strept. uberis,*

Actinomyces pyogenes, Pseudomonas aeruginosa and other *staphylococcus sp.* (El-Balkemy, *et al.*, 1997 and Anwer, *et al.*, 2003).

From the contagious, *Staph. aureus* seemed to be the predominant organisms causing subclinical mastitis (Kader, *et al.*, 2002). It may predispose the herd to infection by coliform or other pathogens (Jain, 1979 and Ibtisam, *et al.*, 1993). *Streptococcus agalactiae* lives in milk and in the mammary gland, but can survive only for a few hours outside of the mammary gland (Refai, 1988 and Phuektes, *et al.*, 2001).

Persistence of *E.coli* within the mammary environment was the cause of recurrent quarter *E. coli* mastitis and its spread among other quarters and cows during the milking process (Bradley and Green, 2001).

The greatest problem in the treatment and control of mastitis is emergence of drug resistance by pathogenic bacteria (Brown and Scassera, 1990 and Jha, *et.al.*, 1994). The pattern of drug resistance continues to change in a particular area depending upon various epidemiological factors and indiscriminate use of antibiotics (Choudhury and Narayan, 1984).

Due to economic and public importance of sub-clinical mastitis, the present work was aimed to detect sub-clinical mastitis and determine the predominant contagious and environmental pathogens causing frequently recurrent sub-clinical mastitis in dairy Holstein-Friesian cows using the most available media for isolation and determination of antibiogram of the most prevalent bacterial isolates.

MATERIALS and METHODS

The present study was conducted on 110 Holstein-Friesian dairy cows with a farm complaint of recurrent mastitis, in Assiut Governorate. All milk samples were screened by Modified Whiteside Test (W. S. T.) (Murphy and Hanson, 1941) and California Mastitis Test (C. M. T.), using Delaval Mastitis Test, 3804101, Poland, (Schalm, *et al.*, 1971).

For bacteriological examination, ten ml of fresh milk samples from 37 cows which showed sub-clinical mastitis positive reaction (individual sampling), as a pooled milk samples of the four quarters in a sterile screw capped vials, were collected aseptically (Rosenberger, 1979). Milk samples were centrifuged at 3000 rpm for 20 minutes, then a loopfull from milk sediment was streaked onto Azid blood agar plate (Cruickshank, *et al.*, 1975) and a loopfull also was inculated into nutrient broth (Diffco), broth 10% Nacl, MacConkey broth (Oxoid) and modified EC-medium (Diffco No.7197405), with novobiocin 2%, for enrichment

of *E. coli* O_{157}, Okrend, *et al.* (1990). The previously inoculated tubes were incubated at 37°C for 24 hours. From the incubated tubes, loopfulls were streaked onto the surface of the nutrient agar, blood agar with 5% sheep blood, mannitol salt agar (BBL), MacConkey agar (Oxoid) and Sorbitol MacConkey agar, for isolation of *E. coli* O_{157}, (Diffco No. 094729/004) plates (Finegold and Martin, 1982; Quinn, *et al.*, 1994 and Heuvelink, *et al.*, 1996 and 1997). The inoculated plates were incubated aerobically at 37°C for 24 hours.

The suspected colonies were identified morphologically by Gram's stain and biochemically confirmed according to Quinn, *et al.* (1994), using catalase activity, coagulase test as well as novoboicin (30 mcg) and polymixin-ß sulphate (300 u) sensitivity tests for identification of *Staphylococcus* spp. As well as identification of *Streptococci* spp. Was done by catalase test, haemolytic activity, sodium hippurate hydrolysis, aesculin hydrolysis on blood agar with 0.1% aesculin, growth in 6.5% Nacl broth, growth on MacConkey agar, Sorbitol, lactose fermentation and Bacitracin, 0.04 unit susceptibility.

Enterobacteriace and *E. coli* O_{157} (*E. coli* O_{157} colonies on Sorbitol MacCokey agar were sorbitol negative and appeared pale in colour as compared with bright pink sorbitol positive colonies produced by *E. coli* and other enteric pathogens, Farmer and Davis, 1985), identified biochemically by conventional IMVIC (Indole, Methyl red, Voges proskauer and citrate utilization) test, motility, triple sugar iron agar (TSI) inoculation and sorbitol, raffinose and cellobiose fermentation, according to (Quinn, *et al.*, 1994 and DeBoer and Heuvelink, 2000).

Antibiogram of the recovered isolates was adapted using antimicrobial susceptibility testing by disc diffusion standard technique according to Bauer, *et al.* (1966), Finegold and Martin (1982) and Quinn, *et al.* (1994). The isolated strains were tested against 10 antibiotics (ciprofloxacin 5 mcg, cepholexin 30 mcg, gentamycin 10 mcg, kanamycin 30 mcg, lincomycin 2 mcg, neomycin 30 mcg, oxytetracyclin 30 mcg, rifamycin 30 mcg, streptomycin 10 mcg and sulbactam 10 mcg plus ampicillin10 mcg), (Bioanalyse-Egypt).

RESULTS

From the obtained results, out of 110 tested milk samples, 35 (31.82%) showed positive C.M.T. results which were true positive as they all revealed positive bacterial isolation. Modified Whiteside Test revealed 37 (33.64%) positive cases (beside the 35 true positive, there

were two false positive cases). The results of bacteriological examination and the antibiogram pattern of the prevalent isolates to different antibiotics are shown in Tables 1, 2 and 3.

Table 1: The frequency percentage of the single and mixed infection in pooled milk samples.

	Single infection	Double infection	Triple infection	Quadruple infection
No.	1	7	20	7
%	2.86	20	57.14	20

Table 2: Bacterial species recovered from sub-clinical mastitis milk of Holstein-Friesian cows.

Bacterial species	No.	Frequency %
Contagious organisms (no.=37)		35.92
-*Staph. aureus*	17	16.50
-*Strept. agalactia*	11	10.68
-*Corynebacterium spp.*	8	7.77
-*Strept. dysgalactia*	1	0.97
Environmental organisms (no.=66)		64.08
-*Enterobacter aerogenes*	13	12.62
-*Enterococcus fuecalis*	11	10.68
-*E.coli* other than O_{157}	11	10.68
-*Strept. equi* subsp.*zooepidemicus*	7	6.80
-*Staph. saprophyticus*	5	4.85
-*Staph .intermedius*	4	3.88
-*Ctirobacter spp.*	3	2.91
-*Proteus spp.*	2	1.94
-*Strept. Pyogenes*	2	1.94
-*E.coli* O157	2	1.94
-*Hafnia alvei*	2	1.94
-*Serratia marcescens*	1	0.97
-*Proteus vulgaris*	1	0.97
-*Salmonella*	1	0.97
-*Shigella*	1	0.97
Total	103	100

Table 3: The percentage of in vitro antimicrobial susceptibility pattern of the most frequent isolates against different antibiotics.

Isolated micro-organisms	No. of the tested isolates	% of sensitive tested strains									
		Ciproflox-acin	Gentamycin	Kanamycin	Lincomycin	Neomycin	Oxytetra-cyclin	Rifamycin	Strepto-mycin	Cepho-lexin	Sulbact-am +Ampi-cillin
Staph. Aureus	17	100	82.35	17.65	0	29.41	47.06	41.18	29.41	17.65	29.41
E. coli	13	100	69.23	61.54	0	61.54	23.08	15.38	15.38	61.54	30.77
Entero-bacter aerogenes	13	100	69.2	15.38	0	7.69	30.77	7.69	30.77	100	30.77
Enterococcus faecalis	11	100	81.82	54.55	0	27.27	27.27	9.09	54.55	27.27	9.09
Strept. Agalactiae	11	100	100	63.6	18.18	36.36	72.7	18.18	36.36	45.45	9.09
Corynebacterium spp	8	100	75	50	0	37.5	37.5	12.5	12.5	25	12.5
Strept. equi subsp. *zooepi-demicus*	7	100	100	57.14	28.57	57.14	28.57	14.29	14.29	57.14	0
Staph. Sarophyticus	5	100	100	20	0	20	60	40	60	20	20
Strept. pyogenes	2	100	50	50	50	50	50	50	50	50	0
Total overall	87	100	80.84	43.32	10.75	36.32	41.88	23.15	33.7	44.89	15.74

DISCUSSION

Sub-clinically infected cows are cyclic shedders of organisms and cycle through low and high shedding patterns of pathogens during lactation. In addition cows suffering from sub-clinically mastitis show no sings, secrete apparently normal milk for long time during which infected animals act as potential reservoir for the responsible causative organisms and spread infection among neighboring animals in the herd (Mohamed, *et al.,* 1993). In the present study results revealed that sub-clinical mastitis in Holstein-Friesian cows were 31.82% by C. M. T. Among Friesian dairy cows, sub-clinical mastitis ranged from 5.5% (Zahid, 2004), 18.5% (Rahman, *et al.,* 1997) up to 67% (Nahed Wahba, *et al.,* 2005). The sub-clinical mastitis incidence varied widely due to changing management conditions and different diagnostic tests used (Radostits, *et al.,* 2000). Since the C.M.T. field test is dependable and reliable perfect test in good agreement with bacteriological results (El-Gaml, 1989 and El-Balkemy, *et al.,* 1997), it appeared to agree 100% with bacteriological isolation in the present work and proved its superiority than modified Whiteside test which detected false positive results, 2 milk samples. False positive of Whiteside test is documented (Nahed Wahba, *et al.,* 2005).

As shown in Table (1), the incidence of mixed infection was 97.14% and single infection was 2.86%, this finding reflects an idea about level of environmental bacterial contamination in the herd and demonstrates the complexity of the disease. In addition *Staph. aureus* may predispose the herd to infection by coliforms or other pathogens (Jain, 1979 and Ibtisam, *et al.,* 1993).

In the present work, shown in Table (2), the frequency percentage of contagious bacteria causing sub-clinical mastitis was 35.92% (*Staph. aureus*, 16.50%; *Strept. agalactia*, 10.68%; *Coryneb.* spp, 7.77% and *Strept. dysgalactia,* 0.97%. The contagious organisms are well adopted to survive in the udder and usually establish mild subclinical infection for long duration (National Mastitis Council, 1987; Mohamed *et al.,* 1993; El-Khodery and Hoedemaker, 2005 and Abdel-Khalek and El-Sherbini, 2005) and can spread from infected quarters to other quarters (Smith and Hogan, 1995; Harmon and Langlois, 1986; Bramley, *et al.,* 1996 and El-Balkemy, *et al.,* 1997).

Staph. aureus and *Strept. agalactiae* are commonly isolated from sub-clinical mastitis (Abou-Zaid and Bahout, 1993; Ahmed and Azza, 2001; Abdel-Khalek and El-Sherbini, 2005 and Hanaa, *et al.,* 2005),

where *Staph. aureus* commonly produce long-lasting infections as it developed sophisticated system to avoid phagocytosis and intra-cellular killing by neutrophils or macrophages (Vanfurth and Van Zwet,1986).

The results obtained in Table (2), revealed that the frequency percentage of environmental bacteria was 64.08%, this result indicated the poor milking hygiene, exposure of the tend end to the environmental pathogens. The environmental bacteria which may cause mastitis usually originate from the surrounding environment including air, soil, water, bedding material, faecal matter, milking man and milking utensils (Anwer, *et al.*, 2003). The portal of entry into mammary gland for Gram-negative bacteria is the teat canal. Once in the gland, bacteria must utilize available substrates in the mammary secretion to replicate and evade host defenses (El-Mahronki, *et al.*, 2006).

The most familiar environmental pathogen, *E. coli*, is widely documented to be a sub-clinical mastitis pathogen (Ahmad, *et al.*, 1991; Todhunter, *et al.*, 1991; Abou-Zaid and Bahout, 1993; Kader, *et al.*, 2002; Anwer, *et. al.*, 2003; Awad and Abeer, 2003 and Moussa, *et al.*, 2006). Its persistence within the mammary environment was of the recurrent quarter *E. coli* mastitis and its spread among other quarters and cows during the milking process (Bradley and Green, 2001).

In the present work identifying of *E. coli* O_{157} was performed by enriching in mEC- medium with novobiocin followed by streaked onto Sorbital MacConkey agar. These media were the efficacious sensitive media for enrichment and isolation of *E. coli* O_{157}, *E. coli* O_{157} colonies was appeared pale in colour (sorbitol negative) on sorbitol MacConkey agar as compared with bright pink sorbitol positive colonies produced by *E. coli* (Farmer and Davis, 1985 and Heuvelink, *et al.*, 1997). Concerning to the biochemical reactions of *E. coli* O_{157} were typical as *E. coli* with exception of sorbitol fermentation (Doyle and Schoeni, 1987). *E. coli* O_{157} was negative for sorbitol, cellubiose and reffinose fermentation in present work. The failure of fermentation of these sugars were the main biochemical differenation of *E. coli* O_{157} from other *E. coli* species (Varnam and Evans, 1991 and De Boer and Heuvelink, 2000). As shown in Table (2) incidence of *E. coli* O_{157} was 1.94%. Moussa, *et al.* (2006) recorded that *E. coli* O_{157} was 3.12% from examined milk samples of sub-clinical mastitic cows.

Enterobacter aerogenes, Proteus vulgaris, Serratia marcescens and Salmonella spp. were identified to be as environmental mastitis pathogens (Ahmad, *et al.*, 1991 and Todhunter, *et al.*, 1991).

Identification of the causative organism and sensitivity testing beside culling of untreatable cows are very important for control of sub-clinical mastitis, so in the present study, the prevalent bacteria isolates were tested for antibacterial sensitivity pattern as shown in Table (3). The obtained results revealed the most effective antimicrobial agent all over the study was ciprofloxacin, followed by gentamycin with susceptibility 100% and 80.84%, respectively. Similar results were obtained by Wadhwa, *et.al.*, 1996; Ahmed and Azza, 2001; Abd El-Hafeez, 2002; Kader, *et al.*, 2002; Abdel-Khalek and El-Sherbini, 2005 and Gad El-Said, *et al.*, 2005.

From the obtained results, it was concluded that recurrent sub-clinical mastitis in Holstein-Friesian dairy cows was mostly caused by mixed infection either contagious or environmental pathogens. Persisted contagious organisms facilitated the inters of environmental pathogens intra-mammary which complicated the problem initiated the recurrences going towards high incidence and bacterial antibiotics resistance.

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