Bacteria implicated with environmental mastitis with special reference to *Escherichia coli*, concerning the biosecurity requirements in dairy farms.

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Abstract
Bovine mastitis represents a disease of high incidence in dairy cattle herds worldwide. Present study was conducted on 2 farms in 2 provinces (Kafr El-Sheik and Dakhlia) with subsequent 5 times visits to assess the magnitude of implementation of measures that given to farms in the first visit to control environmental mastitis . A total of 1286 animals were implicated in this study from 143 animals with clinical mastitis and results obtained revealed 182 quarters were positive to bacteriological examination , *E.coli* was the predominant one (89.56%) followed by extensive differences in prevalence from *Streptococcus dysaglactive* (8.79%) and coagulase negative *S. aureus* (1.65%) , *E. coli* isolates discovered were subjected to serodiagnosis by slide agglutination test and results obtained showed that 130 strains could be identified serologically. They belonged to 9 different serotypes, meanwhile 33strains were untypable by available antisera. The present work was directed to find the correlation between the virulence factors. Out of 63 *E. coli* strains that were tested for their serum resistance activity 138 (84.61%) were able to survive in serum , 68 out of 163 *E. coli* strains showed Congo red binding activity with a total incidence 41.71% . The term invasive *E. coli* referred to those strains of *E. coli* which were able to induce keratoconjuctivitis in eyes of Guinea pigs **,** 65 *E. coli* strains were found to be invasive with an incidence of 39.8% . SDS-PAGE was done for some of *E. coli* isolates discovered from clinical mastitic cases and finally evaluation of different bio security measures (data sheet) in farm animals.

Key words: *E.coli, Streptococcus dysaglactive, S. aureus, keratoconjuctivitis, coagulase negative.*

Introduction

Bovine mastitis represents a disease of high incidence in dairy cattle herds worldwide. It causes considerable economic losses due to decrease in the quality and quantity of milk production, as well as in the cost of treatment, veterinary services and waste **(Dego *et al*., 2002).**

Mastitis is an inflammation of the mammary gland, it occurs as a result of the introduction and multiplication of pathogenic microorganisms in the udder. Bacteria are the main cause of disease and the route of infection is usually through the teat canal which can be classified as contagious or environmental causes **(Philpot and Nickerson, 1991).**

Mastitis management practices intended to control mastitis caused by Gram-negative bacteria often were formulated with the assumption that these bacteria comprised a homogenous group with shared phenotypic characteristics and sources of contamination. More recent findings have revealed that Gram-negative bacteria are a more hetorogenous set of pathogens than previously recognized and the variability among strains may help to explain why management practices intended to control the disease have been minimally successful. **(Pankey *et al*, 2010).**

The bacterial causes of bovine mastitis can be classified as environmental (*E*scherichia coli, *S*treptococcus dysgalactiae, *S*treptococcus parauberis, and *S*treptococcus uberis) or contagious (*S*taphylococcus aureus, *S*treptococcus agalactiae and some *M*ycoplasma species) depending on their primary reservoir **(Smith, 1996).**

Coliforms are the main environmental mastitis pathogens including *E. coli*, Klebsiella spp, Enterobacter aerogens) **(Hogan and Smith, 2003)** where *E. coli* is the most important environmental mastitis pathogen due to its higher incidence **(Friedman et al., 2004 and Cursons *et al*., 2005).** Determinants of mastitis belong to host (resistance, nutrition and stress), environment (housing conditions) and infectious agent (virulence and number of invading pathogen) **(Burvenich *et al.,* 2003 and Mehrzad *et al*., 2004).**

The purchase of replacement heifers and cows are frequently the origin of bacterial mastitis outbreaks in previously free herd. So it is recommended that the purchased cows and heifers should be quarantined and tested from bacterial mastitis before permission to the regular herd **(Ayling *et al.*, 2004).**

Dairy farm expansion and the associated increase in numbers of employees cause major changes in the responsibilities of dairy managers. On small dairy farms most management and labor activities are carried out by just a few people or by one individual. As a farm expands, management responsibilities tend to concentrate with one or a few managers and additional people are hired to take on the majority of the daily production labor **(Feterow, 2000).**

As more employees are added, managers must find better ways to ensure that employees are performing high quality work. The set of practices that managers use to ensure quality employee performance is known as human resource management (HRM). Everything that managers do to recruit, select, train, communicate with, evaluate and terminate employees is included in HRM **(Dessler, 2003).** Human resource management includes practices that managers use to organize, structure and monitor production processes so that employees can more fully understand the process and the impact of their work on results.

SDS-PAGE has become one of the best tools for identification of different strains of bacteria. This technique had been widely applied to study the proteins of all Gram negative bacteria. The bacterial cell proteins especially outer membrane protein that have important role in the virulence of these organisms **(Hancock, 1991 and Amira, 2008).**

The present study was focused mainly to discuss the role played bacteria causing environmental mastitis via the supplementary control measures, In addition, to help to identify areas potential risk on farms the so called , "biosecurity measures in dairy farm" and to how extent it affects the prevalence of environmental mastitis.

Materials and Methods

Collection of samples:

A total of 1286-quarter samples collected from 143 buffaloes showing signs of mastitis (79 animals from Kafr El-Sheik province , in addition to 64 animals from farm in Dakhlia province) with subsequent 5 times visits to assess the magnitude of implementation of measures that given to farms in the first visit.

The udder, teats and hands of milkers were washed and disinfected and samples were collected in separate sterile container and transported to the laboratory with minimum delay in ice box.

Cultivation of milk samples

Milk samples were incubated aerobically at 37°C for 24 hours then centrifuged at 3.000 rpm for 20 minutes.

The cream and supernatant fluid were discarded. A loopfull from the sediment was streaked onto the surface of blood agar, nutrient agar MacConkey's agar, EMB agar, mannitol salt agar and Edward's agar. The inoculated plates were incubated at 37°C for 24-48 hours. Suspected colonies, appearing on different media were subcultured, purified and preserved in semisolid agar for further identification.

Identification of the isolates

Pure colonies were described for their morphological characters, colonial appearance and haemolytic activity. Suspected colonies were picked up and examined microscopically by Gram's stain to observe the morphological arrangement and staining reaction before being transferred into semisolid soft agar to be subjected for further biochemical identification which was carried out according to **Edward and Ewing (1972)** for serological identification of the isolates. Detection of virulence factors of *E. coli* isolates causing mastitis was carried out.

Diagnostic Antisera

Polyvalent and monovalent diagnostic *E. coli* antisera "Denka Seiken Co., LTD" were used for serotyping of *E. coli* isolates.

They included 8 vials of polyvalent and 43 vials of monovalent antisera

Virulence attributes:

Serum resistance test (Barrow and Hill, 1989)

E.coli strains were tested for their ability to survive in bovine sera.

Congo red binding ability (Kerkhoff and Vinal, 1986).

It was used as indicators of virulence among *E. coli*

Invasiveness assay (seleny test) (Wood *et al*., 1986)

The ability of *E. coli* isolates to invade epithelial cells was tested in rabbit eye model "Sereny test" .

Sodium Dodecyl sulphate – polyacrylamide gel Electrophreses (SDS-PAGE) for analysis of whole membrane protein of *E. coli*.

This test performed in four main steps:

A. Propagation of *E. coli* culture.

B. Preparation of *E. coli* antigen.

C. Protein quantitation.

D. Protein banding.

The first two steps were done as described by **Wood *et al.* (1986)**, however the protein banding using SDS-PAGE technique was performed according to **Laemmli (1970) and O`Connor (2006)**.

Biosecurity control measures used in this study (Hogan and Smith,2003)

Control of environmental mastitis is achieved by decreasing teat end exposure to potential pathogens or increasing the animals resistance to mastitis pathogens.

Sanitizers: The use of germicidal sanitizers on teats immediately prior to milking (predipping) . However, the use of post-milking teat antisepsis as a means to control mastitis is unsuccessful.

Dry Cow Therapy: Dry cow therapy significantly reduces new infections by environmental causes of mastitis during the early dry period, but not the week or two before calving.

Lactating Cow Therapy: Cured rates following therapy during lactation are generally about 50 to 60% for the environmental streptococci. Antibiotics approved for lactation therapy are uniformly ineffective against coliform, but cured rates may appear to be as high as 50% due to the short duration of infections.

Milking Machine : Function. Malfunctioning milking machines, that result in frequent liner slips and teat impacts, can increase cases of environmental mastitis.

Udder preparation*:* Milking of cows with wet

udders and teats is likely to increase the incidence of environmental mastitis. Teats should be clean and dry prior to attaching the milking unit. Washing the teats, not the udder, is recommended.

Predipping: Predipping teats with a germicidal teat dip reduces new cases of environmental mastitis during lactation. Extreme caution should be taken to ensure that the teat dip is removed from the teats before milking machine attachment to prevent contaminating the milk.

Immunization*:* Immunizing cows during the dry period with an Escherichia coli J-5 bacterin will reduce the number and severity of coliform clinical cases during early lactation.

Diet: Feeding diets deficient in vitamins A or E, beta-carotene, or the trace minerals selenium, copper, and zinc will result in an increased incidence of environmental mastitis.

Environmental Management*:* Herd environments should be as dry and clean as possible. The environment of the dry, springing and maternity cow is as important as that of the lactating cow.

For human resources management

On farm data collection two detailed questionnaires were prepared that covered information for all aspects of the dairy farm business.

Questionnaire 1. Addressed human resources management, biosecurity, equipment inventory, milking and mastitis, reproduction, housing facilities and feeding management.

Questionnaire 2. Addressed farms management, risk management, dairy quality assurance and included a leadership style assessment tool.

The entire survey took between 1 and 2 hours to complete. All questions were asked in the same order each time. The interviewers asked the questions only as they were written and didn't interpret any information for the producers to prevent any bias.

Continuing Training. Producers were asked to indicate the approximate number of hours of training that employees receive per year, excluding new employees. This was designed to separate those farms that use from those who do not. Respondents were grouped into those that "provided no continuing training" and those that "provided one or more hours of continuing training."

Job Descriptions. Job descriptions are commonly to clarify the duties, roles, and specific responsibilities of employees. Ideally, job descriptions are used as basis for performance appraisal and feedback and to improve communications about specific job responsibilities.

Farm	Area potential of risk	Milk incentive		Continuing training		Job Description		Sanitizer	
		use	don't use	provide	don't provide	Had	non	use	don't use
Kafr El sheikh	-Respiratory symptoms present w/ no dealing - no PPE used - no trained employee - no vaccination for pregnant animals - no house keeping - no dipping w/ disinfectant on the entrance of the farm - low use of decontamination - no room for take off clothes			-	/	-	/	/	-
Dakahlia		-	/	/	-	-	/	/	-

PPE (Personal Protective Equipment)

Results and Discussion

Mastitis is the most frequent and costly disease of dairy cattle. Losses due to mastitis can be attributed to both subclinical and clinical disease. Clinical mastitis losses are generally readily apparent and consist of discarded milk, transient reduction in milk yield and premature culling **(Fetrow, 2000).**

Present study was conducted on 2 farms in 2 province (Kafr El-Sheik and Dakhlia) with subsequent 5 times visits assessment to the magnitude of implementation of measures that was given to farms in the first visit. A total of 1286-animals were involved in this study including 79 animals with clinical mastitis with 100 affected quarters, in addition to 64 animals in the other farm showing signs of mastitis including 82 affected quarters.

Table (1). Total number of examined buffaloes and their quarter milk samples of mastitis.

Locality / Number	Kafr El Sheikh					Dakahlia				
	Total milking	Mastitic animals		Mastitic quarters		Total milking	Mastitic animals		Mastitic quarters	
		No.	%	No.	%		No.	%	No.	%
Animal	710	18	2.5	49	22	576	14	2.4	38	24.4
		19	2.7	51	24		16	2.8	37	23.2
		17	2.4	47	20		12	2.0	34	19.5
		14	2.0	44	18		11	1.9	32	17
		11	1.5	43	16		11	1.9	31	15.9
Total		79	11.2	234	100		64	11.1	172	

A study trial was made to detect the distribution of mastitis among quarters levels and the results showed that the high prevalence was more common to affect four and three quarters (33% , and 28 % respectively) followed by one and lastly two quarters (20.9%% and 18.1%) this consequence are in accordance with **Mosherf (2004) and Aly (2006)** who mentioned that the incidence of three and four quarter infection was comparatively high in clinically mastitic buffaloes. Also **EL-Bakry (2005)** confirmed that mastitis in one and two quarters was more common and that rate of mastitis in one quarters was higher than in two which are similar to that detected in the present study.

Table (2). The total incidence of mastitis in different buffaloes quarters

Locality / No. of Affected Quarters	Kafr El Sheikh		Gharbia		Total mastitic animals	Total mastitic quarters
	Mastitic animal	Mastitic quarter	Mastitis animal	Mastitic quarter		
4 quarters	27	108	21	84	48	192
3 quarters	22	66	17	51	39	117
2 quarters	18	36	14	28	32	64
1 quarter	12	12	12	12	24	24
Total	79	222	64	175	143	397

Table (3). Prevalence of bacteria isolated from mastitic

Species of bacteria	No. of isolates	%
E. coli	163	89.56
Streptococcus dysaglactive	16	8.79
Staphylococcus aureus (coagulase negative)	3	1.65
Total	182	

The present study tries to throw light on the problematic roots of mastitis (disease) involving cooperatively the likelihood pathogen (include the coliforms, the environmental Streptococcal species coagulase negative Stapylococci) that meet certain environment consider the primary habitat of bacteria (faeces, soil, bedding, or water) occur during environmental contact of the teats at milking time or between milkings. In our study lesser variety of bacteria were isolated from the clinical cases of mastitis and *E.coli* was the predominant one (89.56%) followed by wide differences in prevalence of Streptococcus dysaglactive (8.79%) which are one of environmental causes of mastitis and this observation is consistent with the findings of **Wilesmith *et al*. (1986).** The high incidence of *E. coli* isolation meets the finding of **Schukken (2012)** mastitis caused by gram-negative infections is of increasing importance on modern and well-managed dairy farms and dou-

bless, *E. coli* tends to be the most important cause of these gram-negative infections. On the other hand to certain extent our finding agree with that mentioned by **Hogan and Smith (2012)** that the most frequently isolated pathogens were *Staphylococcus aureus, Escherichia coli, Streptococcus uberis*, and coagulase-negative staphylococci and also, the second causative agent was *Streptococcus dysaglactia* by the percentage of 8.79% which are markedly varied from the results obtained by **Mahbub *et al.* (1996)** (31.3%), **Ferguson *et al.* (2007)** (20.6%), **Hala (2010)** (23.7%) the variation between our results and between the other authors described by **Johsi and Gokahal (2006)** to be attributed to different factors including climatic factors, sanitary conditions in the farm and management factors. Coagulase negative *S. aureus* were isolated from only 3 samples with an incidence of 1.65.

E. coli is a risky microorganism due to the great zoonotic importance of some of its strains as O157:H7 **(Doyle, 1991)** and also some cases of *E. coli* mastitis is accompanied by severe symptoms **(Schukken *et al.*, 1989).**

A total of 163 *E. coli* strains isolated from mastitic milk samples were serotyped, 130 strains could be identified serologically. They belonged to 9 different serotyped, meanwhile 33strains were untypable by available antisera.

Table (4). Serotypes of *E. coli* recovered from mastitic milk samples.

E. coli serotype	Total mastitis	
	No.	%
O157	14	8.58%
O153	14	8058%
O55	16	9.81%
O144	16	9.81%
O18	15	9.20%
O78	15	9.20%
O126	13	7.79%
O26	15	9.20%
O1	12	7.36%
Untyped	33	20.24%
Total	163	100

Assessment was done to locate the most major serotypes of *E.coli* isolates in our study and bit by bit reading results obtained we discover that with no doubt there is no main serotype to be focused in as a causative agent, and these results coincide with that mentioned by **Sanchez -Carlo *et al.*, 1984** who emphasized that serotyping of *E. coli* was not of high significance in mastitic cases characterization, same observation had been suggested later (**Zaki *et al.* 2004**).

The present work was directed to find the correlation between the virulence factors. Out of 63 *E. coli* strains that were tested for their serum resistance activity 138 (84.61%) were able to survive in serum and these results coincided with that stated by **Kaipainen *et al.* (2002).**

In this study 68 out of 163 *E. coli* strains showed Congo red binding activity with a total incidence 41.71% in *E. coli* isolates isolated, nearly close results were reported by **Eshak (2002) and Zaki *et al.* (2004)** which were 31.4% and 33%, respectively.

The term invasive *E. coli* referred to those strains of *E. coli* which were able to induce keratoconjuctivitis in eyes of Guinea pigs **(Sereny, 1955 and Silva, 1980).** In this study 65 *E. coli* strains were found to be invasive with an incidence of 39.8% in these results were in contrast to that found by **Mosheref (2004)** who detected only one invasive strain among *E. coli* isolates.

Table (5). Detection of virulence factors of *E. coli* isolated from milk samples

No. of exam *E. coli* Strain	Serum resistance				Congo red				Invasiveness activity (sereny			
	Positive		Negative		Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
163	138	84.61	25	15.4	68	41.71	95	58.2	65	39.8	98	60.1

Sodium dodecyl sulphate polyacrylamide gel electorphoresis SDS-PAGE has been one of the major techniques used for differentiation between protein of different serotypes, through determining marked difference in the number of the obtained bands and molecular weight of each bands. So, SDS-PAGES considered one of the best tools for identification of different strains of *E. coli* and this technique had been widely applied to study the proteins of all Gram negative bacteria. The bacterial cell proteins especially outer membrane protein that have several roles which affect the virulence of these organisms **(Hancock, 1991** and **Ayman, 1999).**

SDS-PAGE of different *E. coli* isolates whole membrane proteins obtained from mastitic milk samples revealed 32 to 37 discrete bands with molecular weights ranged from 11.9 to 130.3 kDa.

The result of SDS-PAGE electrophoresis of *E. coli* isolated from mastitic milk revealed that there were predominate protein bands which was found in most isolates at 13.5, 15.3, 21.9, 24.6, 26.3, 39.1, 46.0, 61.4, 66.5, 69.3, 80.8, 85.7, 89.8, 98.3, 107.2, 112.6 and 118.0 kDa and that reflect that there is a degree of homogenecity between different *E. coli* serotypes examined.

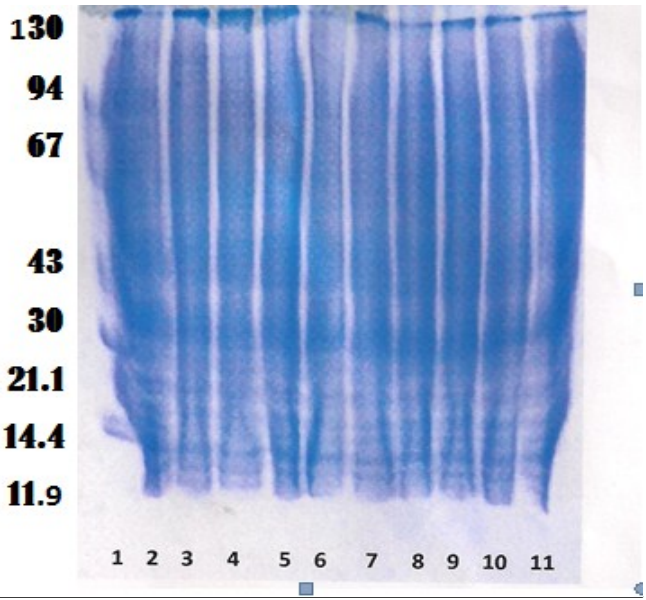
E.coli serotype O55 and typable *E. coli* strain (lane 5 and 10) showed a unique band at 130.3 kDa. *E. coli* serotype O114 and untyped *E. coli* strain (lane 2 and 4) revealed also another special band at 104.6 kDa.

E.coli serotype O18, O157 (lane 8 and 9) showed a special band at 42.2 kDa. While *E. coli* isolates (lane 2, 5 and 8) had a unique band at 47.6 kDa. There were 6 isolates (O55, O18, O78 and 3 untyped *E. coli* strains) that shared a protein band at 32 kDa.

There was another specific band at approximately 94 kDa in which this band was present in 6 of tested 10 *E. coli* strains which represents serotypes O18, O78, O157 and 3 untyped *E. coli* strains.

Specific bands at approximately 42.5 kDa and 18.9 kDa were detected in two *E. coli* strains (isolate no 1 and 9).

Photo. Showing the electrophoretic pattern of *E. coli* isolated from mastitic milk samples.
Lane '1': Low range molecular Marker (cataloge number 161-0304, Pharmacia)
Lane '2': *E. coli* isolate no. 1 (O144)
Lane '3': *E. coli* isolate no. B1 (O78)
Lane '4': *E. coli* isolate no. 3 (untyped)
Lane '5': *E. coli* isolate no. 14 (O55)
Lane '6': *E. coli* isolate no. 9 (untyped)
Lane '7': *E. coli* isolate no. 6 (untyped)
Lane '8': *E. coli* isolate no. 7 (O18)
Lane '9': *E. coli* isolate no. 13 (O157)
Lane '10': *E. coli* isolate no. 9' (untyped)



In the present investigation, SDS-PAGE for ten *E. coli* isolates discovered from mastitic cases were done and produced from 32 to 37 discrete bands with molecular weights ranged from 11.9 to 130.3-kDa.

These results were close to that detected by **Uckun *et al.* (2005)** who made SDS-PAGE of whole cell protein extracts of *E. coli* strains isolated from mastitis cases and found that it produced 26-35 bands with molecular weight ranges from 6.5-200-kDa.

There were predominate protein bands which were found in most isolates at 13.5, 15.3, 21.9, 24.6, 26.3, 39.1, 46.0, 61.4, 66.5, 69.3, 80.8, 85.7, 89.8, 98.3, 107.2, 112.6 and 118.0-kDa and these results reflect that there is a degree of homogenecity between tested *E. coli* strains examined.

Many of the practices and principals of management for reducing the exposure of dairy cows to environmental mastitis pathogens and the common theme for reducing mastitis pathogens in the animals environment is reducing moisture and organic contamination. Frequent manure removal, avoiding overstocking, taking precautions to eliminate stagnant water and providing clean, dry inorganic bedding to lay on are important management considerations. These factors of environmental hygiene transcend stall barns, manure pack barns, open corrals, and pasture systems. The emphasis of control should center on protecting periparturient animals during wet, hot periods of the year when mastitis pathogen growth in the environment is greatest **(Hogan and Smith, 2012)**

We can conclude that, although mastitis result in loss to the producer, at best this loss is confined to medication costs, discarded milk and reduced production as the animals recover. At the worst the production of the animals may be reduced or lost for a lactation with high medical costs, or the animals may suffer permanent damage to her milk producing system or even die. The believe on biosecurity measures or on other words the farm managements are not the embedded philosophy for most of the farms owner or director and this explain that there is not significant change in incidence of mastitis within the five visits to farm. Also our study pointed out that environmental mastitis is more common on farms with excellent control of contagious mastitis. The procedures that reduce the reservoir of contagious mastitis pathogens will not reduce the huge and ever present supply of environmental organisms .Reducing teat-end exposure to environmental pathogensis the goal of an environmental mastitis control program. In addition, clinical coliform cases may negatively impact reproduction in the herd adding to the costs of this disease.

From the main objectives of this study to evaluate the biosecurity data sheet (potential risk) on different it may be the predisposing factors for mastitis explanatory variable are used as training, SOP, job description, milk quality incentive and the using of sanitizer and the results were supporting the expectations.

We are focusing to identify the relationship between these variables used by dairy farms and performance results e.g. it is potential for dairies to use continuous training to increase the productive output from each employee through improvement in skill level by studying SOP we wouldn't have been surprised to find a negative relationship between the use of a milking SOP and somatic cell count (SCC), because SOP are sometimes used to correct poor performance.

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البكتريا المتداخلة مع التهاب الضرع البيئى مع وجود أشارة خاصة لميكروب الايشيرشيا القولونى ودراسة متطلبات الأمن الحيوى فى مزارع الالبان

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الملخص العربى

يمثل التهاب الضرع مرض من الامراض ذات الاهميه الكبيره في قطعان الأبقار الحلوب في جميع أنحاء العالم. وقد أجريت الدراسة في عدد2 من مزارع مقاطعة (كفر الشيخ و الدقهلية).

وقد تم تجميع العينات على 5 مرات متلاحقه لاحقة لتقييم حجم تنفيذ التدابير التي تعطى للمزارع من اول زيارة للسيطرة على التهاب الضرع .كانت الاصابات القولونية من مجموع 1286 عينه عزل عدد 143 عتره من الحيوانات المصابه بالتهاب الضرع . والنتائج التي تم التوصل اليها كشف 182 ربع مصاب و ايجابي للفحص البكتريولوجي، والنسبه الغالبة (89.56٪)للميكروب القولونى يليها الميكروب العقدي(8.79٪) والميكروب المذهب(1.65٪) وقد تم تصنيف 9 عترات سيرولوجيا من العدد الكلى.9 وفي الوقت نفسه كان هناك

عدد 33 عينه لم يتم التمكن من تصنيفهم سيرولوجيا. وجهت هذا العمل للعثور على العلاقة بين عوامل الضراوه المختلفه. من بين 63 سلالات للميكروب القولونى التي تم اختبارها لنشاطهم المقاومة المصل 138 (84.61٪) كانوا قادرين على البقاء على قيد الحياة في مصل الدم، و 68 من أصل 163 السلالات كولاي أظهرت الكونغو الأحمر النشط نسبة 41،71٪ . وقد تم عمل SDs-PAGE لبعض الميكروبات القولونيه المعزوله .ثم تم تقييم مختلف التدابير الامنيه الحيويه لوضع ورقة عمل يتم استخدامها داخل