GLOBAL JOURNAL OF PURE AND APPLIED SCIENCES VOL 16, NO. 4 2010: 381- 389

 ISSN 1118-0579
www.globaljournalseries.com; Email: info@globaljournalseries.com

VARIATIONS IN VIRULENCE OF THREE (3) ESCHERICHIA COLI SEROTYPES CONFIRMED IN EXPERIMENTAL MAMMARY GLAND INFECTIONS

P. A. AKPAN AND J. T. ABRAHAM

(Received 26 May 2009; Revision Accepted 12, January 2010)

ABSTRACT

An experiment was conducted to confirm the pathogenicity of three (3) serotypes of Escherichia coli (E. coli 037, 02a and 109) in mammary glands of experimental cows (cow 105, 107 and 102 respectively). Pathogenicity of the E. coli which is a measure of virulence was observed to vary in the cows. Following inoculation bacterial number peak at 160,000 CFU/ml, 7,6000,000 CFU/ml and 3,600 CFU/ml respectively. Also milk somatic cell count (SCC) were observed to peak at 15,000 x 10^3 cell/ml, 58,700 x 10^3 cell/ml and 360 x 10^3 cells/ml respectively. The time taken for maximum bacterial number and somatic cell count to reach varied. There was leukemia with relative neutropenia in all cases. Typical responses included fever, painful inflammation of glands and gradual weakness of the experimental cows. Time to peak rectal temperature, also varies. The control quarter of teat of each cow infused with 1.0ml of saline showed little or no response. Milk SCC never exceeded 100,000 cells/ml in the control quarter. Systemic effects were little and cows appeared normal externally. E. Coli serotypes varied in virulence with the degree of variation highly determined by the organism tried.

KEY WORDS: pathogenicity, cows leukemia, serotypes

INTRODUCTION

Physical, chemical or biological agents or a combination of these factors can result in inflammation of the mammary gland. Such inflammation is referred to as mastitis. Bacteria have been known as the most capable cause of mastitis. Bushnell (1974) in bacteriological investigation of mastitis reported 90 per cent of mastitis infection caused by gram positive cocci and 5 percent or less by gram negative bacilli, mostly coliform.

Coliform organisms are a large and heterogeneous group of gram negative rods of which *Escherichia coli* is an example. Coliform infections mastitis is often sever and may occasionally end in death (Maegan and Martinko, 2006). Various pathogenic expression are possible on invasion of an organism by bacteria before death. Pathogenic tendency of bacteria and host resistance is a measure of virulence. Among the coliform organisms,
E. coli has the highest incidence of mastitis, but its pathogenesis has not been adequately investigated as has been done in rats and mice. It was necessary to investigate the reaction of cows on invasion of the bacteria which had not been done before. The cost of maintaining cows imposes a limit on the number of experiments that can be performed using cows.

ows are susceptible to *E. coli* infection because *E. coli* exist in the environment of the cows. It was necessary to investigate the reaction of cows on invasion on this bacteria.

MATERIALS AND METHODS

Three (3) cows were obtained from department of veterinary science of Pennsylvania State University, U.S.A. The *E. coli* serotypes were obtained from the department of veterinary science of Pennsylvania state university. Milk sample from e ach quarter of breast teat was tested to ensure that bacteria infection was not already present in their mammary glands and non of the cows had record of coliform infection.

Sequences of examination procedures

A. INITIAL INFECTION TRIAL (COLIFORM FREE TEST)

Cows were held in inverted-v-tier stalls. Each cow was milked at 9.00am and 9.00pm using milking machine. Milk sample from each quarter (breast teat) collected ascetically in separate containers and weighed. Milk somatic cell counts were made by direct microscopic somatic cell-count (DMSCC). (Brazie *et al,* 1968). Milk samples streaked on blood agar plate containing 2% washed bovine red cells were incubated overnight at 37^0C and examined to ensure that bacterial infection was not already present.

B. PREPARATION OF INOCULUM

E. coil strain was transferred from the slant and grown 3 times in trypticase soy broth to ensure rapid growth of the culture. This was incubated for 18hrs at 37^0C. Ten (10) fold serial dilutions in sterile physiological saline were prepared. The number of viable organisms

P. A. Akpan, Department of Biological Sciences Cross River University of Technology – Calabar, Nigeria
J. T. Abraham, Department of Biological Sciences Cross River University of Technology – Calabar, Nigeria

in the 10^7 dilutions were determined by direct microscopic somatic cell-count (DMSCC) in agar pour plates. The dilution that contained the desired number of colony forming units (CFU) (500 CFU) in 1.0ml was determined by plate count (in triplicate).

C. PRE-INOCULATION PREPARATION OF COW

Before inculcation, pulse respiration rates and rectal temperature were taken. Blood sample drawn from the tail vein was taken for total and differential counts. The teat orifice was scrubbed with 70% ethanol. Milk sample was collected from the gland to be inoculated to serve as control.

D. INTRA-MAMMARY INOCULATION OF COWS

The teat orifice was cleansed again with 70% ethanol following milking for control. An estimated 500 CFU of *E. coli* in 1.0ml of physiological saline were infused through the teat canal using teat cannula. The first 3 quarters of mammary gland of each cow in a clockwise direction were infused with 500 CFU of each *E.coli.* The order are as follows cow 105 with *E.coli* 037, cow 107 *E.coil* 02a and cow 102 with *E.coli* 109. The code numbers allotted to cows and *E.coli* serotype were just for identification by the author. Control glands were similarly prepared and 1.0ml of saline were infused. After infusion all teat were dipped in antiseptic solutions and the cows observed for 7 days (168 hours).

Following inoculation pulse and respiratory rates were taken at 3,6,9,12,18 and 24 hrs post inoculation throughout the 7 days. These were done on each quarter. Each quarter experiment lasted for 7 days. During each quarter 7 days observations milk sample was collected at 9.00am and 9.00pm each day.

E. CULTURE OF MILK SAMPLE

The milk samples were cultured as follows:

i. A loopful (0.01ml) of milk sample was streaked on a bovine blood agar plate and incubated overnight.

ii. Colonies resembling *E.coli* in incubated milk sample were inoculated into TS 1 and simmons citrate agar.

III. Reactions consistent with those of *E.coli* in TS 1 and simmons citrate agar were transferred to trypticase soy gar slant.

IV. Agar slants were wax sealed and maintained at room temperature until when needed.

F. (1) NUMERATION OF BACTERIA

ach milk sample in agar was prepared in ten-fold serial, dilutions 10^1,10^2,10^3, 0.1ml of each dilution was incorporated in a trypticase soy agar plates. These were incubated overnight at 37^{o-}C and the colonies counted. Milk somatic cell count was also determined.

(2) ENUMARATION OF BLOOD

Blood leucocytes were counted with an electronic particle counter. Blood smears for differential cell-counts were prepared on microscope slides and stained with wrights stains (Platt, 1969). Approximately 1ml of Blood was transferred to a tube containing 0.2mg disodium ethylene dinitrilotetracetate (EDTA) for further examination.

RESULTS

Typical response observed after inoculation of mammary glands was development of acute mastitis with local inflammation. Also there was alteration in the nature of the gland's secretion and systematic signs including fever and inappetence.

Bacterial colonies cleared quickly from the mammary secretion every 72 hours and were observe to have O antigen in all case.

The response of the cows to each of the *E. coli* strains showed variations in virulence of bacteria. These are stated below

A. RESPONSE OF COW 105 TO *E. COLI* 037

he right front quarter of cow 105 was infused with 500CFU of *E.coli* 037.

Following infusion, bacterial numbers increased through the 9th hour peaking at 160,000CFU/mol at the 12th hour. Milk somatic cell count (SCC) increased slowly through the 12th hour, then more rapidly to a maximum of 15,000 x 10^3 cells/ml at the 24th hour.

fter reaching a maximum, bacteria counts decreased rapidly and at the 48th hour the organism was not detected by the plate count method, but was isolated by pre-incubation of the milk sample before plating. The organism was not recovered thereafter. Milk SCC decreased more slowly from peak value of 15,000 x 10^3 cell/ml.

ectal temperature was elevated at the 18^{th} (103.0^0 F) and 24^{th} (102.4F) hours. In blood there was leukemia beginning at the 12^{th} hour with lowest leukocyte count at the 36^{th} hour. Relative neutropenia as seen in differential leukocyte counts was most evident at 18^{th} and 24^{th} hrs,

otal milk production was substantially reduce at the 24, 36, 48, and 60 hours milkings. Lowest total milk production was at the 36^{th} hour. Minimum milk yield from this quarter was obtained at the 36^{th} hour. By the end of the observation period, total milk yield had returned to about 80 percent.

ilk was serous at the 12^{th} and 18^{th} hours. Clots were first observed in the milk at the 24^{th} hour. Clot persisted in milk of this quarter until the end of the seventh day observation period. Although bacteria were not recovered after the 48^{th} hour.

Bacterial number at this point declined rapidly and were detected only after pre-incubation of 60, 72, and 84 hour milk samples. The response of cow 105 to

E. coli 037 is shown on figure 1 and complete data on table 1.

B RESPONSE OF COW 107 TO *E. coli* 02a

This strain of *E. coli* (02a) was one of high virulence in mouse experiment and elicited a severe response in this cow experiment.

Bacteria numbers reached a maximum of 7,600,000 CFU/ml. cell count increased rapidly to a level of more than 58,700 x 10^3 cells/ml by the 18th hour. Total milk yield was most markedly reduce at the 24th through 36th hour milkings. Rectal temperature rose and reach a maximum of 107 4^0F at the 9th hour.

In blood there was leukemia with relative neutropenia. These changes were evident by the 18th hour and most marked at 48 hours.

The inflammation of teat quarters in this cow caused by *E. coli* 02a was more severe than that of other *E. coli* in other cows. Swelling persisted through 36th hour. Clots were present at 12th hour and persisted through 108 hours.

The response of cow 107 to *E. coli* 02a is shown graphically in figure 2 and complete data is shown in table 2.

C RESPONSE OF COW 102 TO *E. coli* 109

his strain of *E. coli* had the highest LD_{50} (lethal dose) of all serotypes in the mouse experiment. acterial multiplication was limited compared with that in other experiments. Bacterial number reached a peak of 3,600 CFU/ml at the 6th hour and decline thereafter. At the 18th and 24th hours bacteria was recovered only from pre-incubation milk samples.

ilk SCC were very slightly increased at 12 hours through 26 hours. There was an apparently spurious count of about 360 x 10^3 cells/ml at the 36th hour.

here was very little inflammation of mammary gland with no rectal temperature rise and no increase in milk secretion. The response of cow 102 to

E. coli 109 is shown on table 3.

The control quarter of each cow infused with 1.0ml of saline, showed little response.

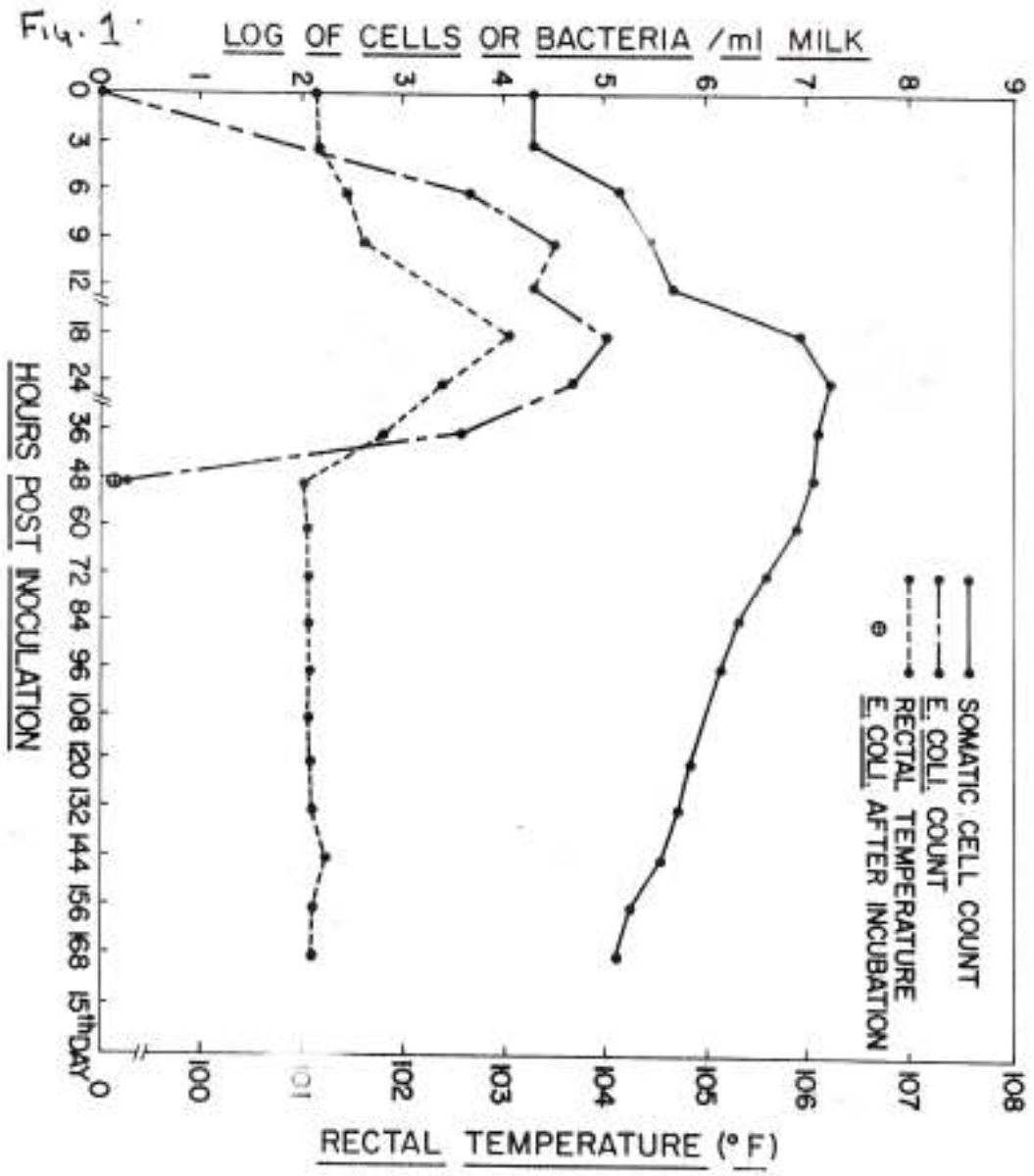
Fig. 1
LOG OF CELLS OR BACTERIA /ml MILK
0 1 2 3 4 5 6 7 8 9
HOURS POST INOCULATION
0 3 6 9 12 18 24 36 48 60 72 84 96 108 120 132 144 156 168 15th DAY
RECTAL TEMPERATURE (°F)
100 101 102 103 104 105 106 107 108
SOMATIC CELL COUNT
E. COLI. COUNT
RECTAL TEMPERATURE
E. COLI. AFTER INCUBATION

Table 1 Responses in Cow 105 following the inoculation of 500 colony-forming units (CFU) of E. coli 037 into the right front quarter. The left front quarter was infused with 1.0 ml sterile saline as a control.

Time Post-inoculation (Hrs.)	Milk Somatic Cells (Cells x 10^{-3}/ml)		E. coli in Milk (CFU/ml)	Blood Leukocytes (Cells/mm^3)	Proportion of Leukocyte Types (%)					Clinical Signs					Milk Yield[1]	
	Inoculated Quarter	Control Quarter			Neutrophils	Eosinophils	Lymphocytes	Monocytes	Others	Rectal Temp. (°F)	Udder	Secretion	Resp./Min.	Pulse/Min.	Inoculated Quarter	Total
0	67.0	33.0	0	9,060	30	11	58	4	3	101.3	N	N	34	64		
3	95.0	421.0	1,600	9,000	33	6	49	1	1	101.2	N	N	34	64		
6	261.0	67.0	400	8,600	36	8	46	7	3	101.4	N	N	46	66		
9	302.0	20.0	2,500	6.000	27	2	51	11	9	101.6	N	N	36	66		
12	483.0	20.0	160,000	5,300	24	3	59	3	11	101.7	H-	S	64	80	1.8	14.1
18	9,000.0	225.0	93,000	4,000	18	3	48	4	16	103.0	H	S	40	96		
24	15,000,0	463.0	5,800	3,000	17	1	61	6	15	102.4	H-	C-	40	76	1.4	7.8
36	12,300.0	260.0	3,000	2,000	29	9	36	11	17	101.5	H-	C-	32	80	0.3	7.6
48	13,170.0	175.0	⊕	6,200	28	13	47	7	6	100.8	N	C+	32	80	0.8	10.0
60	9,760.0	302.0	0	9,200	35	6	50	7	2	100.9	N	C+	28	76	1.6	11.6
72	4,360.0	67.0	0	6,500	29	6	53	3	9	101.1	N	C++	28	66	2.0	13.2
84	1,510.0	300.0	0	5,000	41	11	37	1	13	101.0	N	C++	30	66	2.5	12.6
96	1,280.0	220.0	0	5,200	34	8	66	6	6	101.0	N	C-	24	72	3.0	15.4
108	1,470.0	20.0	0	6,100	26	0	60	3	11	101.0	N	C-	32	68	2.3	11.6
120	2,310.0	54.0	0	5,500	36	0	50	5	9	101.0	N	C-	32	70	3.4	16.2
132	802.0	22.0	0	6,300	30	6	58	2	4	101.2	N	C=	32	72	2.9	14.2
144	604.0	13.0	0	7,600	14	4	64	7	11	101.4	N	C=	34	68	3.0	14.6
156	3,900.0	—	0	7,000	20	11	51	7	11	101.1	N	C=	28	64	3.1	14.3
168	503.0	60.0	0	8,600	21	6	62	8	3	101.0	N	X=	28	64	3.0	14.2

⊕ = E. coli recovered after incubation; N = normal; H = moderately hard; H- = less hard; S = serous; C- = few clots; C= = close to normal; C+ = more clots; C++ = a lot of clots;

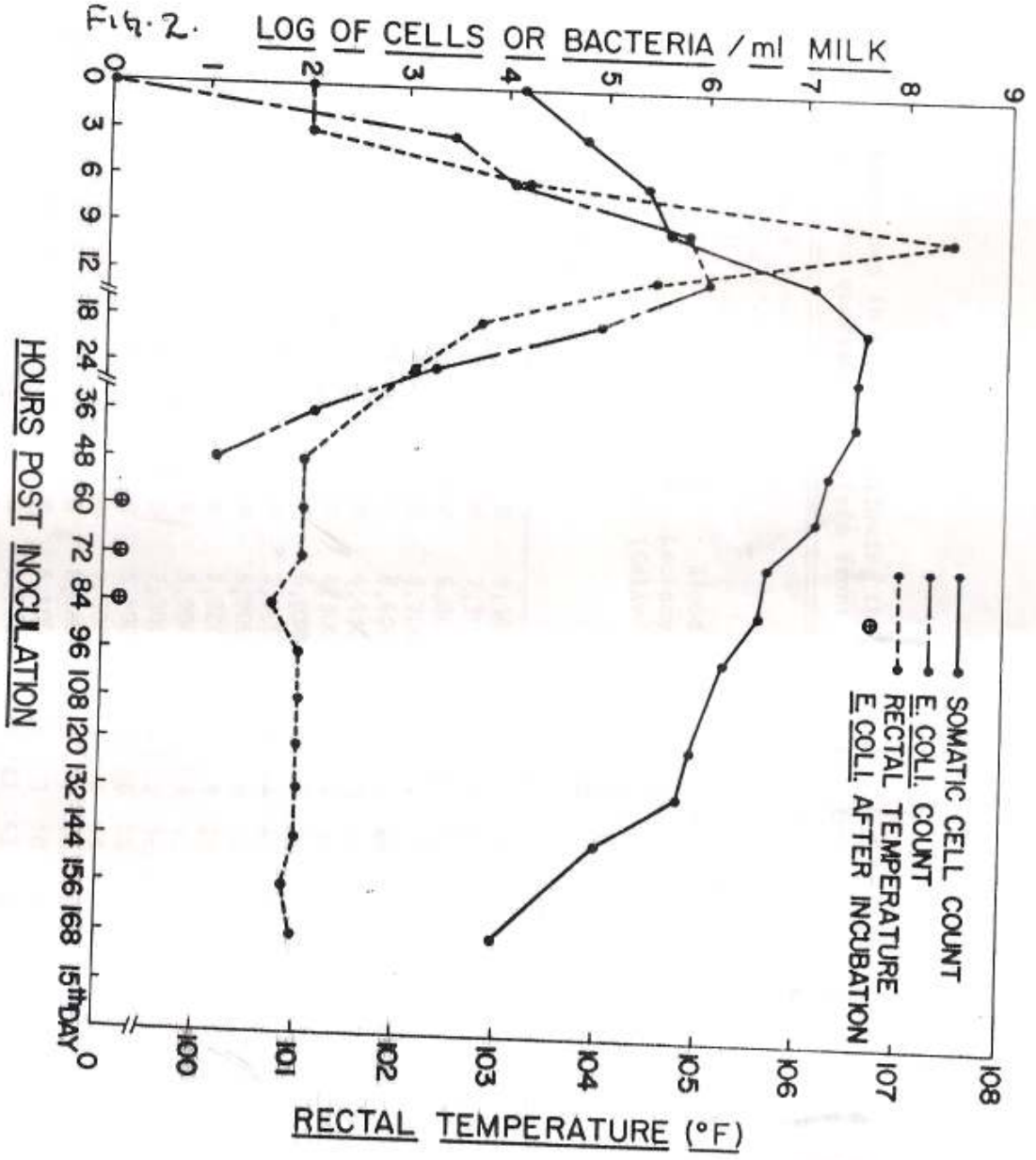
Fig. 2.
LOG OF CELLS OR BACTERIA / ml MILK
0
1
2
3
4
5
6
7
8
9
HOURS POST INOCULATION
0 3 6 9 12 18 24 36 48 60 72 84 96 108 120 132 144 156 168
15th DAY
RECTAL TEMPERATURE (°F)
100 101 102 103 104 105 106 107 108
SOMATIC CELL COUNT
E. COLI. COUNT
RECTAL TEMPERATURE
E. COLI. AFTER INCUBATION

Table 2. Responses in Cow 107 following the inoculation of 500 colony-forming unity (CFU) of E. coli 02a into the right rear quarter. The left front quarter was infused with 1.0 ml sterile saline as a control.

Time Post-inoculation (Hrs.)	Milk Somatic Cells (Cells x 10^{-3}/ml)		E. coli in Milk (CFU/ml)	Blood Leukocytes (Cells/mm^3)	Proportion of Leukocyte Types (%)					Clinical Signs					Milk Yield[1]	
	Inoculated Quarter	Control Quarter			Neutrophils	Eosinophils	Lymphocytes	Monocytes	Others	Rectal Temp. (°F)	Udder	Secretion	Resp./Min.	Pulse/Min.	Inoculated Quarter	Total
0	13.5	--	0	9,300	30	6	50	6	8	101.0	N	N	28	72		
3	64.0	2.7	1,900	7,700	21	4	62	2	11	101.2	N	N	28	72		
6	220.0	--	11,000	6,800	28	6	54	3	1	104.3	N	N	28	70		
9	350.0	13.0	400,000	8,200	21	6	59	11	3	107.4	H	N	76	100		
12	13,600.0	20.0	7,600,000	8,600	18	5	64	4	9	105.6	H	C	36	100	4.6	19.0
18	58,700.0	4.1	100,000	6,430	17	3	34	11	7	102.6	H	C	64	80		
24	39,600.0	4.5	3,700	4,300	31	4	50	3	12	102.4	H	C+	24	64	1.0	11.0
36	32,400.0	47.0	200	6,050	14	9	34	8	3	102.2	H	C+	44	80	1.3	15.6
48	18,600.0	220.0	40	2,600	9	4	42	4	9	100.8	N	C+	28	76	4.4	20.8
60	13,300.0	60.0	⊕	6,700	21	6	50	7	16	101.0	N	C	32	68	4.9	19.2
72	4,300.0	40.0	⊕	2,800	22	9	29	3	5	101.0	N	C	28	80	5.8	22.2
84	4,020.0	27.0	⊕	5,900	31	9	42	7	11	100.0	N	C	40	68	5.4	20.5
X96	2,800.0	27.0	0	7,300	24	2	36	2	10	101.0	N	C	24	76	6.8	23.2
108	2,200.0	47.0	0	5,500	23	6	58	7	6	101.0	N	C	36	76	3.8	16.0
120	2,740.0	20.0	0	9,300	19	8	58	3	12	101.0	N	N	24	76	6.8	24.2
132	1,580.0	6.7	0	6,400	23	2	49	6	8	101.0	N	N	32	60	5.5	21.1
144	1,450.0	6.7	0	6,100	39	3	50	3	5	101.0	N	N	24	64	5.0	22.2
156	139.0	6.7	0	5,900	36	11	42	2	11	100.8	N	N	32	64	5.7	21.7
168	6.0	20.0	0	7,800	32	4	49	6	9	101.0	N	N	32	60	5.0	21.8

⊕ = E. coli recovered after incubation; N = normal; H = moderately hard; C = clots; C+ = more clots; C- = few clots;

Table 3 Responses in Cow 102 following the inoculation of 500 colony-forming units (CFU) of E. coli 0109 into the right front quarter. The right rear quarter was infused with 1.0 ml sterile saline as a control.

Time Post-inoculation (Hrs.)	Milk Somatic Cells (Cells x 10^{-3}/ml)		E. coli in Milk (CFU/ml)	Blood Leukocytes (Cells/mm^3)	Proportion of Leukocyte Types (%)					Clinical Signs					Milk Yield[1]	
	Inoculated Quarter	Control Quarter			Neutrophils	Eosinophils	Lymphocytes	Monocytes	Others	Rectal Temp. (°F)	Udder	Secretion	Resp./Min.	Pulse/Min.	Inoculated Quarter	Total
0	80.5	60.0	0	5,600	30	2	65	1	3	101.4	N	N	48	69		
3	40.2	60.0	1,600	4,900	33	6	59	0	2	101.4	N	N	48	76		
6	160.0	40.0	3,600	6,900	36	6	52	4	5	101.5	N	N	56	76		
9	120.0	77.0	1,100	3,040	21	1	65	5	8	101.5	N	N	60	76		
12	302.0	100.0	500	5,300	26	6	59	2	7	101.8	N	N	54	76	3.1	16.9
18	282.0	110.0	⊕	6,500	24	2	63	4	7	101.8	N	N	56	70		
24	221.0	181.0	⊕	6,100	29	3	47	6	11	101.2	N	N	48	76	3.2	18.1
36	360.0	80.0	0	4,600	31	6	52	3	8	101.3	N	N	60	76	3.0	15.6
48	142.0	60.0	0	6,800	36	3	44	5	12	101.0	N	N	54	76	2.4	15.0
60	80.5	60.0	0	5,200	22	8	58	9	13	101.7	N	N	54	72	3.2	17.5
72	24.0	120.0	0	5,800	31	1	60	1	7	100.8	N	N	52	66	3.0	16.2
84	161.0	100.0	0	7.400	42	3	49	2	4	101.0	N	N	56	68	3.0	14.3
96	280.0	65.0	0	5,200	26	0	65	2	4	101.3	N	N	60	80	3.0	16.4
108	120.0	40.0	0	5,900	28	2	59	3	8	102.4	N	N	56	80	3.0	17.4
120	60.0	4.0	0	5,400	31	1	66	1	1	101.0	N	N	56	80	3.0	16.4
132																
144																
156																
168																

⊕ = E. coli recovered after incubation; N = normal.

DISCUSSIONS

noculation of mammary glands of cows with *Escherichia coli* showed variations in virulence of these bacteria. Virulence denotes ready reaction to the presence of external influences or contact with another body which has little or no compatibility. Thus, *E. Coli* may not be a normal flora in mammary glands of cow.

he response of each cow to a single dose of approximately 500 CFU of *E. Coli* serotype infused into mammary quarter showed systemic and local response which reflects both the virulence of the organism and the resistance of the host. Differences in time taken for expression of clinical signs by, *E. Coli* strains in different cows was observed. The delay between infusion of the inoculum and the onset of clinical signs was more marked with the less virulent strains as shown on tables 1, 2 and 3 respectively.

he local and systemic signs of mastitis including fever, increased heart rate and swollen glands are attributed to the release of endotoxin by the gram negative bacteria. As earlier observed by Caroll*.,* (1983), Schalm *et al.,* (1991), Dugid *et a*l., (1982) and Todar, 2007). Leukemia observed in the study is believed to be due to host sensitized immunity due to invasion of *E. Coli.* Thus great number of leucocytes were released which reduced with time as bacteria number decreased. The time for peak change in leukogram count varied between the 12th and 72nd hours of infection. Because of the wide variability of both mean total leukocyte and differential counts, no relationship was found between virulence of the strain and the changes in blood leukocyte. These changes were observed in coliform mastitis by Schalm *et al.,* (1995). Reduction in number of total white blood cells is due to movement of neutrophils in large numbers from blood to milk. The blood picture returned to normal as new generations of neutrophils became available to peripheral blood from the bone marrow cells.

eduction in milk yield from the infection quarter was most marked during the peak of infection. Bacterial and somatic cell counts in milk and body temperature tended to be higher in infections by more virulent strain as observed in mice (Akpan and Ikpeme, 2005).

omatic cell count at peak for the serotype (109) with highest virulence of *E. coli* in mouse does not always produce equal virulence in cows. This may be attributed to differences in genetic bases of mouse and cow. However, this needs to be investigated.

ACKNOWLEDGEMENT

We are grateful to the Pennsylvania state university, USA for providing the funds , the cows and other facilities for this study. We deeply appreciate the cooperation of Prof. R. E. Eberhart, and . L. Bortree of the department of veterinary sciences the Pennsylvania State University P.A. USA.

REFERENCES

Akpan, P. A and Ikpeme, E. U., 2005. pathology of Experimental *Escherichia Coli* infections in mice: A laboratory animal model for research in mastitis. (Research Paper in Press).

Brazie, A. R., Jasper, D. E., Levowltz, P., Newbould, F. H., Postie, D. S., Schultze, W. D., Smith, J. W. and Ullman, W. W. (National mastitis council subcommittee on sceening test, 1969. Direct microscopy somatic cell count. Milk and Food Technol 13 pp. 1-350.

Bushnell, R. B., 1974. where are we on coliform mastitis. *ational mastitis council. Inc. Annual meeting*. Pp. 69.

Caroll, E. J., Schala, O.W. and Lasmanis, J., 1983. Experimental coliform *(Aerobacter aerogenes)*; mastitis, distribution of whey protein during the early acute phase. *Journal of Dairy Science*, 46

Duguid , J .P Marmion, B. P. and Swain, R. H. A., 1980.Mackie and McCartney . Medical Microbiology 13th edition, (vol.1). William Clowes (Beccles) Ltd. Beccles and London. Pp 1-113.

Madigan, M. T and Martinko, J. M., 2006. Brock Biology of Microorganisms. Pearson. Pp 8-13

Platt, W. R., 1969. Color Atlas and Textbook of Hematology, 6th edition. Lippincott company. Philadelphia. Pp 1-335.

Schalm, O. W, Caroll, E. J. and Jain, N. C., 1991. Bovine Mastitis. Lea ands Febiger Philadelphia. pp .1-250.

Schalm, O. W,. Jain, N. C. and Caroll, E. J., 1995. Veterinary Hematology, 5th .edition. Lea Febiger, Philadelphia. Pp.1-300.

Todar, K., 2007. Pathogenic E. coli. University of Wisconsin. Pp 11-30.