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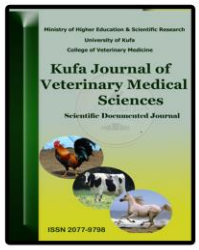

Extractionof biofilm produced by *Escherichia coli* that are isolated from animals infected with diarrhea and study its protective role

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Abstract

This study was conducted to detect the ability of *E.coli*isolated from diarrhea to produce biofilm and protection against diseases cause by these bacteria compared with whole cell sonicated antigen.

One hundred two fecal samples (52 fecal samples from cows, calves and 50 fecal samples from sheep,goat) were collected from College of veterinary medicine-university of Baghdad, College of Agriculture-university of Baghdad, Dora zone and Abu-Ghraib zone. Samples were cultured on MacConkeyand Eosin Methylene Blue agar and after purification of cultured bacteria, biochemical tests, API 20 E System andRapID^{TM} ONE System kit were done.

Results showed that 91 out of 102 fecal samples have the characteristics belong to*E.coli*,the ability of theseisolates to produce biofilm were detected and the results showed that 38 out of 49 *E.coli* isolates from fecal samples of cow produce biofilm (77.55%)and 39 out of 42 *E.coli* isolates from fecal samples of sheep produce biofilm (92.85%) with different thickness ranged between (0.2-2)mm, while 11 isolates from 49 fecal samples of cow and calves and 39 isolates from 42 fecal samples of sheep had not produce biofilm.

The results showed that protein concentration of biofilm was 92 mg/ml for one isolate and 70 mg/ ml for the whole sonicated antigen of the bacteria that produce biofilm.

Three types of bacterial antigens were prepared as follow; Whole cell sonicated antigen (WCA) of biofilm producer *E.coli*, biofilm extract antigen (biA) with protein concentration 3.5 mg/ml and biofilm extract antigen (biA) with protein concentration 14 mg/ml, then these antigens were injected in 50 healthy White BALB mice.

Results showed that survival time of animals in the immunized group with biofilm antigen of high protein concentration (14 mg/ml) was longer (652.8 hrs) than animals immunized with whole sonicated antigen of protein concentration 3.5 mg/ml antigen (378 hrs) and than the group with biofilm antigen of protein concentration 3.5 mg/ml (513.6 hrs) , heavy bacterial isolation were recorded in the internal organs of the immunized infected animals at (12-48) hours post infection, while moderate bacterial isolation at day 30 post infection.

Histopathological examination showed large abscess which caused adhesion of liver with stomach and spleen with stomach and pancreas surround by dense cellular fibrous tissue, the result showed that all animals of control positive group,5 animals of group injected with(WCA) produce biofilm,3 animals of group injected with (biA) 3.5

mg/ml and 1 animal of group injected with (biA) 14 mg/ml were died during 12-48 hours post infection, acute suppurative reaction were seen in internal organs of animals died at 12-24 hours post infection, granulomatous lesions were seen in most internal organs of animals in group 2 , 3 and 4 at day 30 post challenge, while mild inflammatory reaction was recorded in most internal organs of group 4 at day 30 post challenge.

Introduction:

E .coli is a normal inhabitant of the intestines of most animals and humans. Some *E.coli* strains can cause a wide variety of intestinal and extra – intestinal diseases , such as diarrhea ,urinary tract infections , septicemia , mastitis and neonatal meningitis (1). The formation of bacterial biofilms of *E.coli* in a host in general seems to be based on current evidence to a large extent an intra cellular event (2).

The diseases caused by a particular strain of *E. coli* depend on distribution and expression of many virulence determinants such as biofilm formation, adhesion ,production of haemolysin, enterotoxin, shiga toxin ,endotoxin and capsules formation (3).

Microbial biofilms were extremely complex microbial ecosystems consisting of microorganisms attached to a surface and embedded in an organic polymer matrix of microbial origin , As well as microbial components , non – cellular materials such as mineral crystals , corrosion particles, clay or silt particles , or blood components , may also be found in the biofilm matrix , so a biofilm has to be kept general and thus may be redefined as " microbial cells immobilized in a matrix of extra cellular polymers acting as an independent functioning ecosystem, homeostatically regulated" (4).

Diarrheal diseases were major problem in third world countries which are responsible for death of millions of people and animals each year, diarrhea is an alteration in normal bowel movement and it is characterized by an increase in the water content, volume, or frequency and decrease of dry matter of feces (5, 6, 7). It can be either acute or chronic (8).

Diarrhea accounts of 46% of calves and lambs mortality (9).the most common causes of acute diarrhea are bacterial and viral infections (10, 11).Infections with *E. coli* being one of the major causative agents (12).

Materials and Methods:

MacConkey and Eosin Methylene Blue agar were used for growth and isolation of *E.coli*from fecal samples were collected from (sheep , goats , cows and calves) suffering from diarrhea .Morphological , cultural and biochemical tests in addition to API20 E system and RapID^{TM} ONE System were used for the diagnosis of isolates .

Bacterial Virulence test:

After all confirmatory tests were done to identify the bacteria, suspension of bacterial isolate that produced biofilm with the highest thickness was used in preparation of challenge dose which was made by harvesting a cultured plate and 0.3 ml was injected in 5 mice to examine its virulence.

Preparation of challenge dose:

The preparation of the bacterial suspension and the counting were made using McFarland's tubes according to procedure described by (13). This bacterial suspension containing 1.5×10^8 cfu/ml of *E.coli*was injected intra peritoneal (I/P) by insulin syringe.

Experimental design:

Fifty mice were divided equally into five groups, ten mice in each :

Group1:10 mice as positive control.

Group2:10mice immunized with 0.25 ml (WCA) from *E.coli*produce biofilm S/C with protein concentration (3.5 mg\ml) at day zero as primary dose followed by booster dose after 15 days.

Group3:10 mice immunized with0.25 ml (BiA) S/C with protein concentration (3.5 mg /ml) as a primary dose followed by booster dose after 15 days.

Group 4:10 mice immunize with0.25 ml(Bi A) S/C with protein concentration (14 mg/ ml) as a primary dose followed by booster dose after 15 days.

Group 5: 10 mice (negative control) were injected with 0.25 ml of PBS S/C and repeated after 15 days.

After 30 days the first four groups were challenged with 0.5 ml(1.5 $x10^8$ CFU/ml) of *E.coli*I/P and group five were injected with PBS I/P, animals which were died after 12 , 24, 48 and 72 hours were examined for bacterial isolation. After 30 days the remained animals were sacrificed for its bacterial count and histological study of Intestine, liver and spleen.

Results and Discussion:

Microscopical , cultural characteristics , biochemical and confirmatory tests :

Results of bacterial isolation showed that out of total 102 fecal samples, 91 fecal samples showed positive results for the presence of *Escherichia coli* after culturing one on EMB agar.Table (1).

The results showed difference between the various zone in the growth of fecal samples on Macckonky agar and EMB with different morphological characteristics of *E.coli* on different media, after incubation at 37°C for 24 hours. Isolated bacteria appeared as gram negative rods, non spore forming under light microscopic lense and these results agreed with that recorded by (14). The red /pink color on MacConkey agar that occurred was due to the utilization of the lactose that available in the medium with surrounding areas of precipitated bile salts, While EMB agar was used for selection and isolation purposes, and was considered as a rapid and accurate method for distinguishing *E. coli* from other gram-negative pathogens. The visible colonies appeared as green metallic sheen that indicates vigorous fermentation of lactose and acid production which precipitates the green metallic pigment .This result agrees with (15).TheBiochemical identification results showed that these isolates belong to *E.coli* were positive for catalase, indol,methyl red test and motility test with the ability to produce gas on kligler iron test while gave negative results for oxidase, simmon citrate test, urease test and vogesproskaure test . Table (2).

The results of Api 20 E system and RapID^{TM}ONE System confirmed that these 91 isolates out of 102 fecal samples were belong to *E.coli* .

Biofilm production results

The test was done by using Christensen tube method (16).Results of *E.coli* isolated from fecal samples of cow,calves showed that out of 49 *E.coli* isolates, 38 appeared the ability to produce biofilm (77.6 %) while 11 (22.4%) isolates gave negative results, as showed in table (3).While 39 out of 42 *E.coli* isolated from fecal samples of sheep and goat produced biofilm (92.9%) and only 3 isolates (7.1%) showed negative results for this test as

in table (4).These results are in agreement with (17) who showed that 46 of total 56 isolate of *Staphylococcus aureus* produced biofilm (82.14%) while only 10 isolates gave negative results (17.85) ,(18) demonstrated that 32 of 35 *S. aureus* isolates produced biofilm (91.42) These results are in agreement with these in the present study .while (19) found a lower percentage (12%) of biofilm-positive producer strains in 92 bovine strains and this is disagreement with the current study.

The results showed that these isolates differed in its biofilm producing efficiency , the thickness of biofilm which measured in these isolates ranged between (0.2-2)mm , (20) found that the thickness of biofilm produced by *pseudomonas aeruginosa* ranged between (1.1-6.5)mm,these results are disagreed with the present ones, while (17) showed that thickness of biofilm produced by *S.aureus*ranged between (0.2-1.5) mm and this is in agreement with the present results Table(5,6).

Biofilm thickness results

Table (1): Results of isolation of *E.coli* from fecal samples:

city of collected samples	Total No.	Positive samples on	
		MacConkey agar	EMB agar
Abu- Ghraib zone	35	33	32
College of veterinary medicine -university of Baghdad	28	26	23
College of agriculture - university of Baghdad	17	16	16
Dora zone	22	20	20

Table (2): Results of cultural, microscopical, and biochemical tests:

Biochemechal test	Catalase	Oxidase	Simmon Citrate	Urease	Kliger Iron	Indol	Motility	Methyl Red	Vogas - Proskaure	Gram Stain	Cell Morphology
Result	(+) ve	(-) ve	(-) ve	(-) ve	Yallow / Yallow with gas	(+) ve	(+) ve	(+) ve	(-) ve	(-) ve	Smooth , glassy , rosy pink on MacConkey with appearance of green metalic sheen on EMB

Table (3) Results of biofilm production of different *E.coli*isolates from fecal samples (cow):

No. of isolates	Result	No. of isolates	Result	No. of isolates	Result	No.of isolates	Result
1	-	14	-	27	+	40	+
2	+	15	+	28	+	41	+
3	+	16	+	29	+	42	-
4	-	17	+	30	+	43	+
5	+	18	+	31	+	44	+
6	+	19	+	32	+	45	+
7	+	20	+	33	+	46	+
8	+	21	+	34	+	47	+
9	-	22	-	35	+	48	-
10	+	23	+	36	+	49	-
11	-	24	+	37	-		
12	+	25	+	38	+		
13	+	26	+	39	-		

Table (4) Results of biofilm production of different *E.coli*isolates from fecal samples (sheep):

No. of isolates	Result	No. of isolates	Result	No. of isolates	Result	No.of isolates	Result
1	+	12	+	23	+	34	+
2	+	13	+	24	+	35	+
3	+	14	+	25	+	36	+
4	+	15	+	26	+	37	+
5	+	16	+	27	+	38	+
6	+	17	-	28	+	39	+
7	-	18	+	29	+	40	+
8	-	19	+	30	+	41	+
9	+	20	+	31	+	42	+
10	+	21	+	32	+		
11	+	22	+	33	+		

+ produce biofilm - not produce biofilm

Table (5) Thickness of biofilm produced by *E.coli* isolates from fecal sample of cows:

No. of isolate	Thickness of biofilm (mm)	No. of isolate	Thickness of biofilm (mm)
2	0.2	26	0.2
3	2	27	0.2
5	0.7	28	0.2
6	1.5	29	0.5
7	0.8	30	1
8	0.2	31	0.9
10	0.2	32	0.2
12	1	33	1
13	0.4	34	0.5
15	0.2	35	0.2
16	0.2	36	0.9
17	1.5	38	0.5
18	1	40	0.5
19	1	41	0.6
20	0.4	43	0.5
21	0.5	44	0.3
23	0.3	45	0.7
24	0.5	46	0.2
25	2	47	0.4

Table (6) Thickness of biofilm produced by *E.coli*isolates from fecal samples of sheep:

No. of isolate	Thickness of biofilm (mm)	No. of isolate	Thickness of biofilm (mm)
1	0.8	24	2
2	0.2	25	0.3
3	0.9	26	0.2
4	0.2	27	2
5	0.8	28	0.4
6	0.4	29	0.2
9	0.3	30	0.5
10	0.6	31	0.2
11	0.2	32	0.5
12	0.3	33	0.2
13	0.2	34	0.2
14	0.2	35	0.6
15	0.4	36	0.5
16	0.2	37	0.6
18	0.4	38	0.2
19	0.2	39	0.6
20	0.4	40	0.3
21	0.2	41	0.2
22	0.4	42	0.3
23	0.2	43	0.6

This study showed that all non immunized infected animals were died during 12,24 hours particularly at 24 hours post infection (6 animal died) with mean survival time 19.2 hours , also heavy bacterial isolation was obtained from examined organs depending on number of colony grown on EMB agar was done as showed in Table(7,8).

One animal of group immunized with whole cell sonicated antigen were died at 12 hrs post infection ,one animal died at 24 hours and three died at 48 hrs post infection with survival time 378 hours. While immunized animal with biofilm antigen with protein concentration 3.5 mg/ml,two animal died at 24 hours and one animal died at 48 hours post infection with survival time 513.6 hours and heavy bacterial isolation from internal organ of animal of these groups that died during 24-48 hours with moderate bacterial isolation at day 30 post infection Table (9) ,however one animal of immunized group with biofilm of high protein concentration (14 mg/ml) were died at 48 hours with moderate bacterial isolation, the survival time of this group is 652.8 hours.

These results indicated that *E.coli* isolatesin this study induced septic shock in non immunized infected animal and most immunized animal with sonicated antigen of biofilm producing strain,while immunized animal with biofilm with high concentration provide immune response that activate the peritoneal macrophage and destroyed most of inoculated bacteria at site of injection in lag phase of growth.

Table (7): Bacterial isolation (on EMB) from the internal organs of the control positive and immunized mice infected with virulent *E.coli* at 12-48 hours post challenge:

	Hours		
Groups	**12 hrs**	**24 hrs**	**48hrs**
Group 1	+++	+++	0
Group2	+++	+++	++
Group 3	0	+++	++
Group 4	0	0	++

+++ heavy (over than 11 colonies),++ moderate (6-10) colony

Table (8) :Bacterial isolation (on EMB agar) from the internal organs of the immunized mice infected with virulent *E.coli* at 30 days post challenge :

At 30 days post challenge				
Group 2	8×10	6×10^{2}	2×10^{3}	$\times110^{4}$
Group 3	5×10	3×10^{2}	1×10^{3}	0
Group4	3×10	1×10^{2}	0	0

Hours / Groups	12 hrs	24 hrs	48 hrs	72 hrs	720 hrs		Total hrs
Group 1 (positive control) 10 mice	4	6	0	0	0	192/10	19.2
	48	144	0	0	0		
Group 2 (WCA) produce biofilm 3.5 mg/ml 10 mice	1	1	3	0	5	3780/10	378
	12	24	144	0	3600		
Group 3 (bi A) 3.5mg/ml 10 mice	0	2	1	0	7	5136/10	513.6
	0	48	48	0	5040		
Group 4 (bi A) 14mg/ml 10 mice	0	0	1	0	9	6528/10	652.8
	0	0	48	0	6480		

Table (9):Survival time of mice

Results of Histopathological examination:

At 12hours post-infection:

Control positive group (Group 1):

Liver : The histopathological section in the liver tissues at 12 hours post infection with *E.coli* of control positive group showed vacuolar degeneration of hepatocytes (Fig:1) as well as thrombus formation.

Intestine: The main lesion in the intestine of the animals in group 1 at 12 hours post infection with *E.coli*characterized by few inflammatory cells ,infiltration in the lamina properia (LP) and mucosal glands (Fig: 2).

Spleen: The microscopic examination revealed that spleen of animals in group 1 at 12 hours post infection with*E.coli* expressed apoptosis of lymphocytes of white pulp that left multiple spaces filled with cellular debris (Fig: 3).

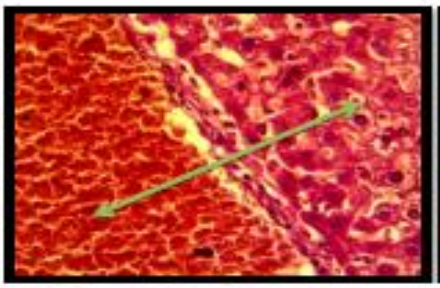

Fig: 1. Histopathological section in the intestine of animal at 12hours post-infection showed vacuolar degeneration of epithelial cells with few inflammatory cells infiltration between mucosal glands (H&E stain 40X).

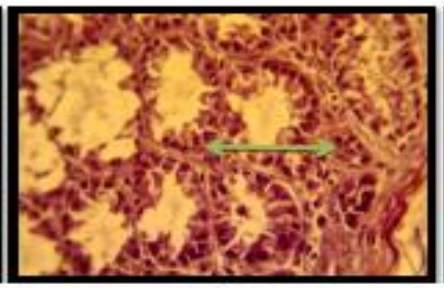

Fig:2. Histopathological section in the intestine of animal at 12hours post-infection showed vacuolar degeneration of epithelial cells with few inflammatory cells infiltration between mucosal glands (H&E stain 40X).

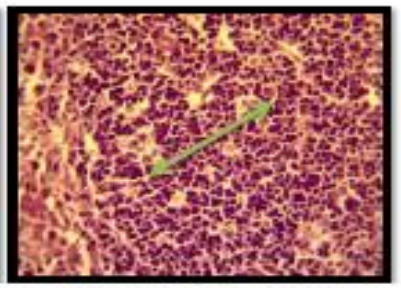

Fig:3.Histopathological section in the spleen of animal at 12hours post-infection showed apoptosis of lymphocytes with space containing cellular debris vacuolar (H&E stain 40X)

Liver: The microscopic section of the liver of animals in group 2 at 12 hours post infection with *E.coli*showed investigated inflammatory cells aggregation in dilated sinusoids (Fig: 4), as well as, focal vacuolar degeneration of hepatocytes with proliferation of kupffer cells(Fig: 4) .

Intestine: The intestine of immunized animal with whole sonicatedAgs of biofilm producing strain with protein concentration 3.5mg\ml at 12 hrs post-infection showed inflammatory cells infiltration in the lamina properia (Fig:5).

Spleen: The main lesions in the spleen of immunized animal with whole sonicatedAgs of biofilm producing strain with protein concentration 3.5mg\ml at 12 hours post-infection consisted from amyloid like substance deposition around hyperplasia of white pulp (Fig: 6).

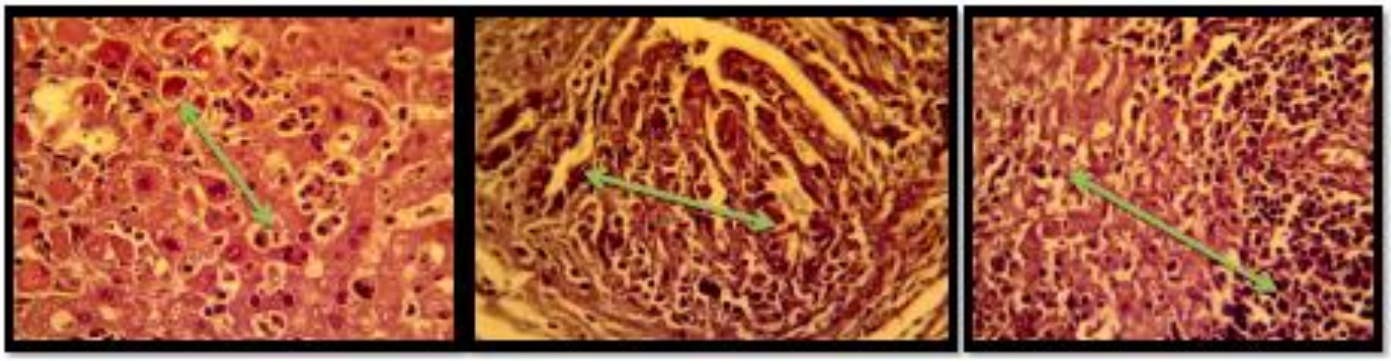

Fig:4.Histopathological section in the liver of immunized animal with whole sonicatedAgs of biofilm producing strain with protein concentration 3.5mg\ml at 12 hours post-infection showed inflammatory cells aggregation in dilated sinusoids(H&E stain 40X)

Fig:5.Histopathological section in the intestine of immunized animal with whole sonicatedAgs of biofilm producing strain with protein concentration 3.5mg\ml at 12 hours post-infection biofilm protein3.5mg\ml showed inflammatory cells infiltration in the lamina properia(H&E stain 40X)

Fig:6.Histopathological section in the spleen of immunized animal with whole sonicatedAgs of biofilm producing strain with protein concentration 3.5mg\ml at 12 hours post-infection showed amyloid like substance deposition around hyperplasia of white pulp(H&E stain 40X)

24 hours post – infection:

Control positive group (group 1):

Liver : the histopathological changes in the liver tissues after one day post infection with *E.coli* showed thrombus in the blood vessels , dilated and congested of central veins and sinusoids with neutrophils in there lumen , necrosis of hepatocytes which characterized by pyknotic or disappearance of their nuclei (fig: 7).

Intestine: The histopathological section in the intestine of control positive group at 24hours post-infection showed hypertrophy and hyperplasia of goblet cells with inflammatory cells infiltration between mucosal glands (Fig: 8).

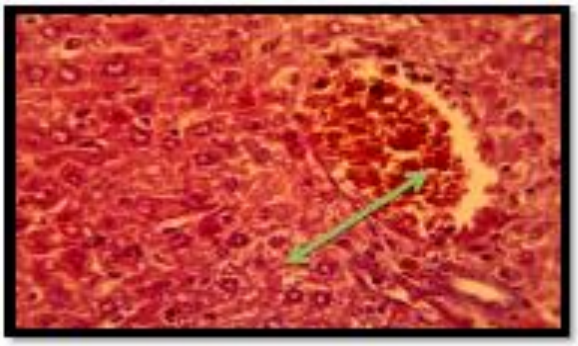

Fig:7Histopathological section in the liver of control positive group at 24hours post-infection showed thrombus in the blood vessels , dilated and congested of central veins and sinusoids with neutrophils in there lumen(H&E stain 40X)

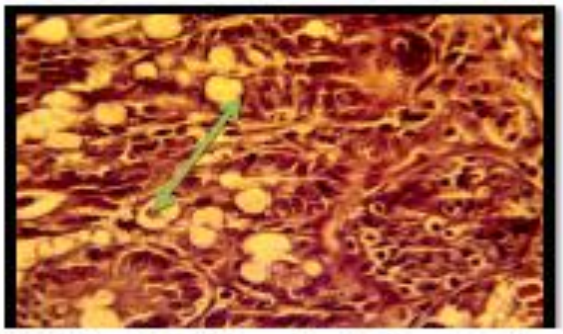

Fig:8.Histopathological section in the intestine of control positive group at 24hours post-infection showed hypertrophy and hyperplasia of goblet cells with inflammatory cells infiltration between mucosal glands(H&E stain 40X)

Animals immunized with whole sonicated antigen (WCA) of biofilm producing *E.coli* isolate (group 2):

Histopathological examination in the liver tissues of animals immunized with whole sonicated antigen (WCA) of biofilm producing *E.coli* isolate at 24 houurs post - infection showed aggregation of mononuclear cells around congested blood vessels and sinusoids as well as parenchyma of the liver with kupffer cells proliferation (Fig:9)

The spleen revealed proliferation of lymphocytes in the periarteriolar sheath (Fig: 10).

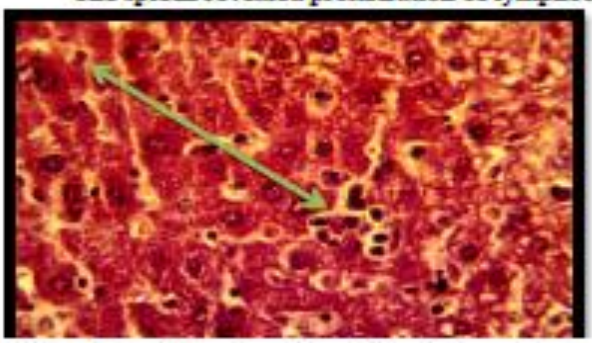

Fig:9.Histopathological section in the liver of immunized animal with whole sonicatedAgs of biofilm producing strain with protein concentration 3.5 mg /ml at 24 hours post-infection showed aggregation of mononuclear cells in the liver parenchyma and proliferation of kupffer cells(H&E stain 40X)

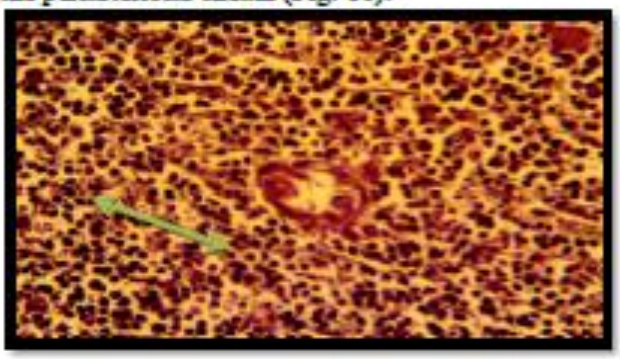

Fig: 10.Histopathological section in the spleen of immunized animal with whole sonicatedAgs of biofilm producing strain with protein concentration 3.5mg\ml at 24 hours post-infection showed proliferation of lymphocytes in the periarteriolar sheath(H&E stain 40X)

Animals immunized with biofilm antigen (bi A) with protein concentration 3.5 mg/ml (group 3):

Liver: The histopathological section in the examined organ of Animals immunized with biofilm antigen (bi A) with protein concentration 3.5 mg/ml at 24 hours post - infection expressed severe vacuolation and necrosis of hepatocytes (Fig: 11).

Spleen: The histopathological examination of immunized animal with biofilm producing strain with protein concentration 3.5mg\ml at 24 hours post-infection revealed that spleen expressed moderate hyperplasia of white pulp (Fig: 12).

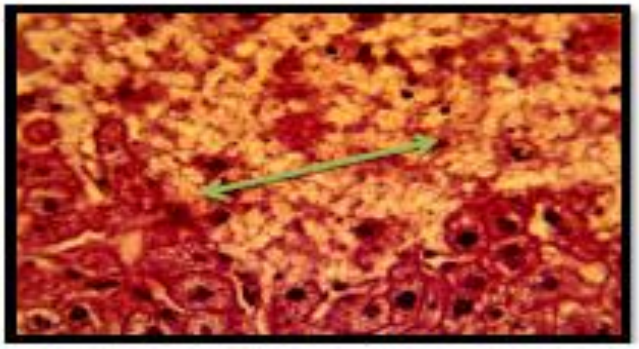

Fig:11.Histopathological section in the liver of immunized animal with biofilm producing strain with protein concentration 3.5mg\ml at 24 hours post-infection showed severe vaculation and necrosis of hepatocytes ↔ (H&E stain 40X)

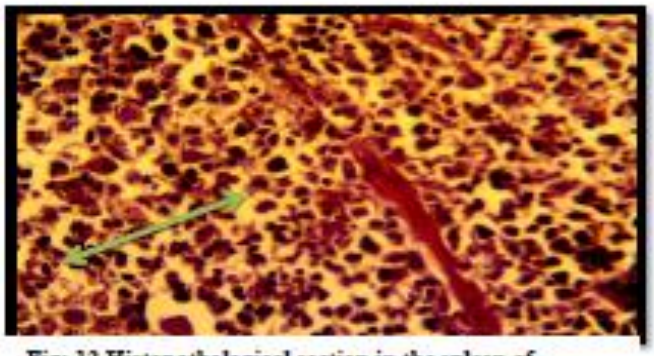

Fig: 12.Histopathological section in the spleen of immunized animal with biofilm producing strain with protein concentration 3.5mg\ml at 24 hours post-infection showed depletion of white pulp(H&E stain 40X)

Animals immunized with whole sonicated antigen (WCA) of biofilm producing *E.coli*isolate (group 2):

Histopathological section in the liver of Animals immunized with whole sonicated antigen (WCA) of biofilm producing *E.coli*isolate at 48 hours post infection revealed fatty changes and vacuolar degeneration of the hepatocytes and inflammatory cells aggregation in portal are(Fig:13) while no clear lesions were recorded in the intestine (Fig: 14) .

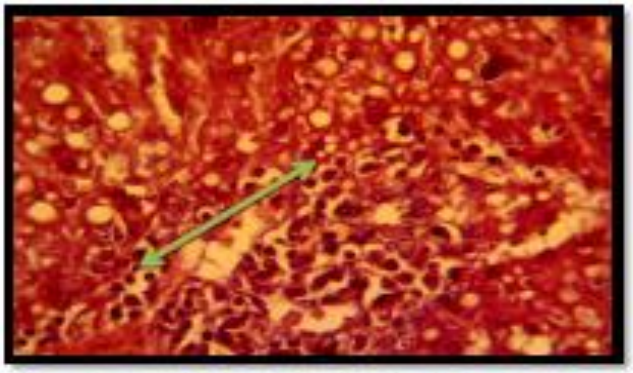

Fig:13.Histopathological section in the liver of immunized animal with whole sonicatedAgs of biofilm producing strain with protein concentration 3.5mg\ml at 48 hours post-infection showed fatty changes and mononuclear cells aggregation in the portal area ↔ (H&E stain 40X).

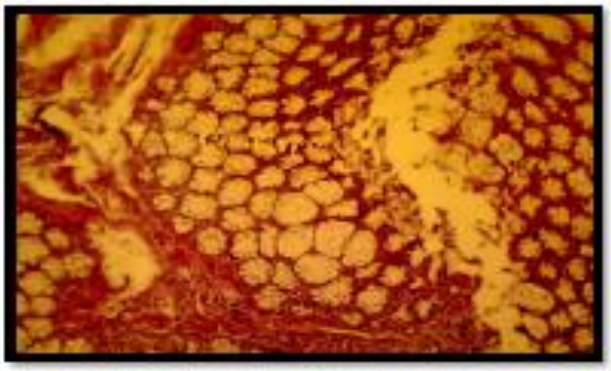

Fig:14.Histopathological section in the intestine of immunized animal with whole sonicatedAgs of biofilm producing strain with protein concentration 3.5mg\ml at 48 hours post-infection showed no clear lesions (H&E stain 40X).

Animals immunized with biofilm antigen (bi A) with protein concentration 3.5 mg/ml (group 3):

Liver:Histopathological section in the liver of animals immunized with biofilm antigen (bi A) with protein concentration 3.5 mg/ml at 48 hours post-infection expressed marked foamy enlargement of hepatocytes (Fig:15).

Fig:15.Histopathological section in the liver of immunized animal with biofilm Ag: of biofilm producing strain with protein concentration 3.5mg\ml at 48 hours post-infection showed foamy enlargement of hepatocytes ⟷ (H&E stain 40X).

Animals immunized with biofilm antigen (bi A) with protein concentration 14 mg/ml (group 4):

Liver: The main lesions in the liver ofanimals immunized with biofilm antigen (bi A) with protein concentration 14 mg/ml at 48 hours post-infection characterized by foamy enlargement of hepatocytes with occluded of the sinusoids (Fig: 16).

Intestine:Histopathological section in the intestine of immunized animal with biofilm producing strain with protein concentration 14mg\ml at 48hours post-infection showed moderate mononuclear cells infiltration in the lamina properia (LP) (Fig: 17).

Spleen: The microscopic sections of spleen of immunized animal of biofilm producing strain with protein concentration 14mg/ml at 48hr post-infection showed moderate proliferation of lymphocytes in the periarteriolar sheath were the main lesions in the spleen (Fig: 18).

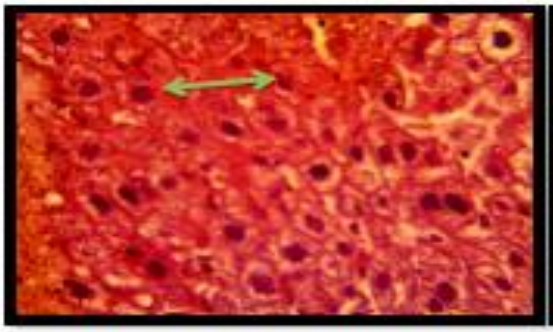

Fig:16.Histopathological section in the liver of immunized animal with biofilm producing strain with protein concentration 14mg\ml at 48hr post-infection showed foamy enlargement of hepatocyte (H&Estain 40X). ⟷

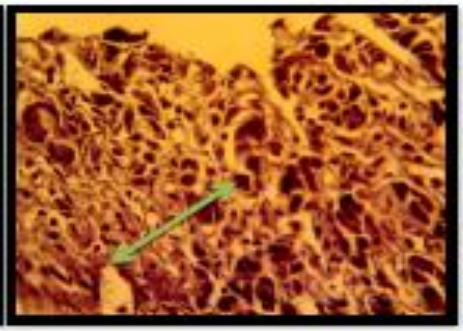

Fig: 17.Histopathologicalsection in the intestine of immunized animal of biofilm producing strain with protein concentration 14mg/ml at 48hr post-infection showed moderate mononuclear cells infiltration in the lamina properia (LP)(H&Estain 40X). ⟷

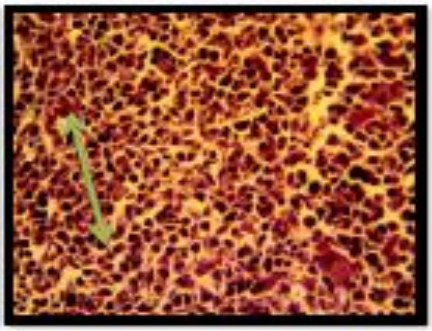

Fig:18.Histopathological section in the spleen of immunized animal of biofilm producing strain with protein concentration 14mg/ml at 48hr post-infection showed moderate proliferation of lymphocytes in the periarteriolar sheath (H&Estain 40X).

Animal sacrificed at day 30 post – infection:

Animals immunized with whole sonicated antigen (WCA) of biofilm producing *E.coli*isolate (group 2):

Liver: Histopathological section of the liver of animals immunized with whole sonicated antigen (WCA) of biofilm producing *E.coli*isolate at day 30 post – infection expressed mono nuclear cell aggregation around congested blood vessels (fig: 19) .

Intestine: the microscopic section in the intestine of immunized animal with sonicatedAgs of biofilm production strain with protein concentration 3.5 mg/ml at day 30 post – infection showed no clear lesion except hyperplasia of goblet cell mucosal gland and mononuclear cell aggregation between mucosal gland and hyperplasia of goblet cell (fig: 20).

Spleen :The spleen of immunized animal with sonicatedAgs of biofilm production strain with protein concentration 3.5 mg/ml at 30 day post- infection showed hyperplasia of whit pulp (fig :21).

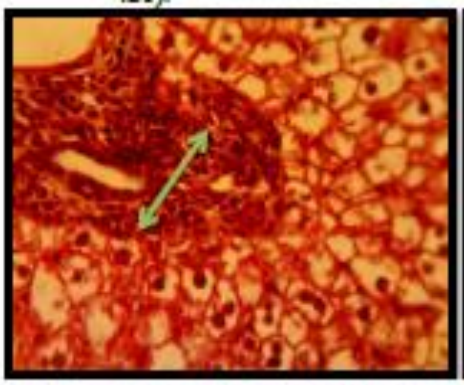

Fig: 19. Histopathological section in the liver of immunized animal with sonicatedAgs of biofilm production strain with protein concentration 3.5 mg/ml at 30 day post- infection showed mono nuclear cell aggregation around congested blood vessels (H&E 40X)

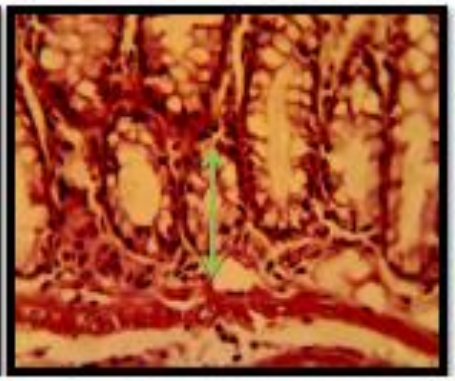

Fig: 20. Histopathological section in the intestine of immunized animal with sonicatedAgs of biofilm production strain with protein concentration 3.5 mg/ml at 30 day post- infection showed mononuclear cell aggregation between mucosal gland and hyperplasia of goblet cell gland (H&E)

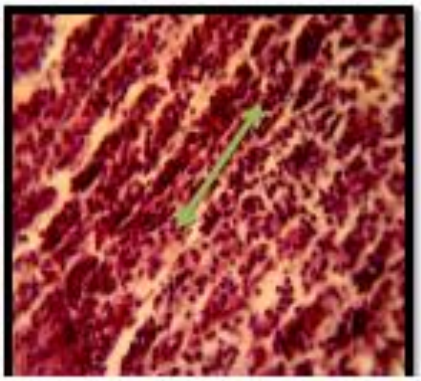

Fig: 21. Histopathological section in the spleen of immunized animal with sonicatedAgs of biofilm production strain with protein concentration 3.5 mg/ml at 30 day post- infection showed hyperplasia of whit pulp (H&E)

Animals immunized with biofilm antigen (bi A) with protein concentration 3.5 mg/ml (group 3):

Liver :the microscopic section of the liver of of immunized animal with sonicatedAgs of biofilm production strain with protein concentration 3.5 mg/ml at 30 day post- infection showed multiple granulomatous lesion scattered in the liver parenchyma (fig :22).

Intestine : The histopathological changes in the intestine of Animals immunized with biofilm antigen (bi A) with protein concentration 3.5 mg/ml at 30 day post- infection showed inflammatory cell infiltration particularly macrophage , lymphocyte between mucosal gland (fig : 23).

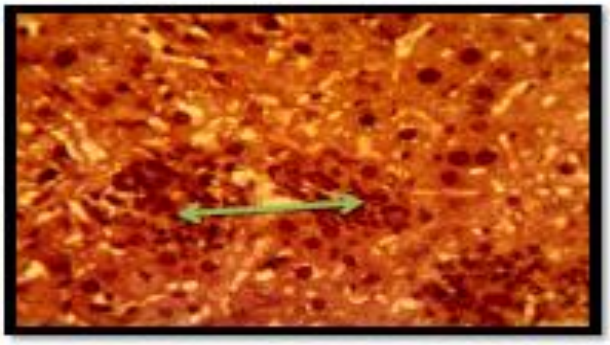

Fig: 22. Histopathological section in the liver of immunized animal with biofilm Ags of biofilm production strain with protein concentration 3.5 mg/ml at 30 day post- infection showed multiple granulomatous lesion scattered in the liver parenchyma (H&E 40X).

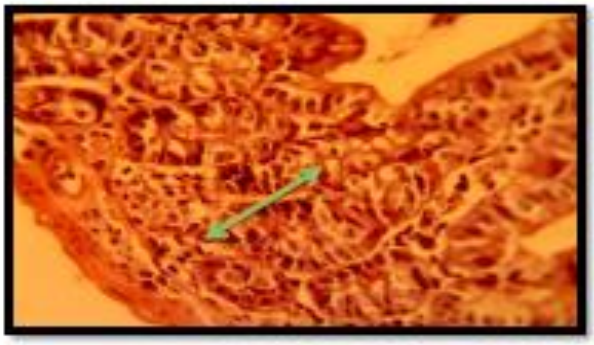

Fig: 23. Histopathological section in the intestine of immunized animal with biofilm Ags of biofilm production strain with protein concentration 3.5 mg/ml at 30 day post-infection showed inflammatory cell infiltration particularly macrophage , lymphocyte between mucosal gland (H&E 40X).

Animals immunized with biofilm antigen (bi A) with protein concentration 14 mg/ml (group 4):

Liver: the microscopic examination demonstrated that the lesion in the liver ofanimals immunized with biofilm antigen (bi A) with protein concentration 14 mg/ml at day 30 post – infection showed mononuclear cell aggregation in one side of the blood vessels in the portal area (fig: 24).

Intestine :The examined organ of immunized animal with biofilm Ags of biofilm production strain with protein concentration 14 mg/ml at 30 day post- infection showed mononuclear cell infiltration between mucosal gland (fig :25)

Spleen : The histopathological section of the spleen of immunized animal with biofilm Ags of biofilm production strain with protein concentration 14 mg/ml at 30 day post- infection showed proliferation of lymphocytes in the periarteriolar sheath (fig : 26).

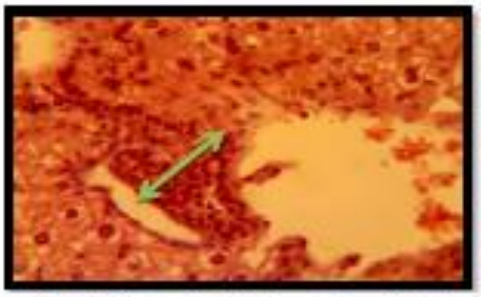

Fig: 24. Histopathological section in the liver of immunized animal with biofilm Ags of biofilm production strain with protein concentration 14 mg/ml at 30 day post-infection showed mononuclear cell aggregation in one side of the blood vessels in the portal area (H&E 40X).

Fig: 25. Histopathological section in the intestine of immunized animal with biofilm Ags of biofilm production strain with protein concentration 14 mg/ml at 30 day post- infection showed mononuclear cell infiltration between mucosal gland (H&E 40X).

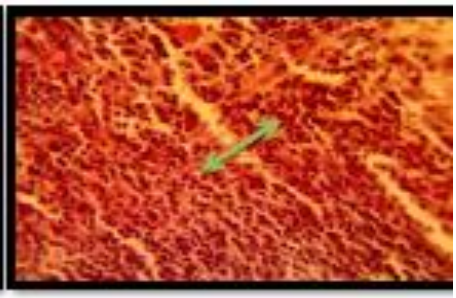

Fig: 26. Histopathological section in the spleen of immunized animal with biofilm Ags of biofilm production strain with protein concentration 14 mg/ml at 30 day post- infection showed proliferation of lymphocytes in the periarteriolar sheath (H&E 40X).

Control negative group (group 5):

There is no lesion in liver and intestine of animals in the control negative group at day 30 post – infection (fig: 27, 28).

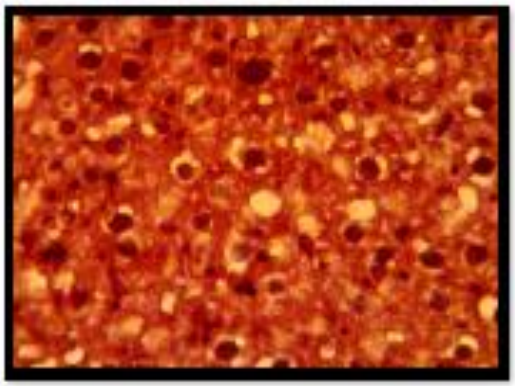

Fig: 27. Histopathological section in the liver of the control negative group at day 30 post – infectionshowed the normal structure of the liver (H&E 40 X).

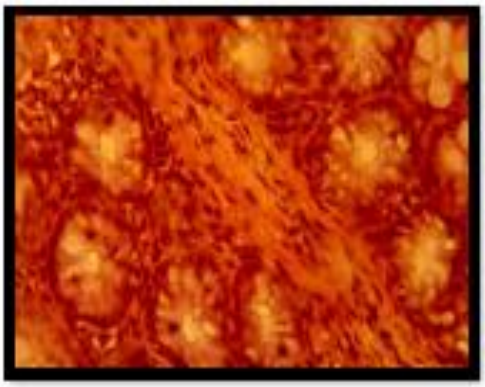

Fig: 28. Histopathological section in the intestine of the control negative group at day 30 post – infection showed the normal structure of the intestine (H&E 40 X).

The histopathological examination showed that the dead mice were exposed to highly virulent microorganisms that induced septic shock with multi-organs dysfunction, thrombus formation and death during 12 hrs. Immunized animals with Whole cell sonicated antigens that died during 12 hrs. post infection expressed lesions proximately similar to the control positive group in addition to variable degrees of inflammatory cells aggregation of hepatocytes with proliferation of Kupffer cells in the liver, inflammatory cells infiltration in the lamina properia in the intestine and hyperplasia of the white pulp of the spleen also these results was given the indication that WCA *Ecoli*Ags alone provide partial protection against virulent *E coli* infection while animal immunized with biofilm strengthen the immune responses againstvirulent *E coli* specially highly concentration this result may indicate that antigen used in the present study stimulated immune response with variable degrees according to type of antigen,but this immune response unable to protect the animal from highly virulent challenge dose of *E. coli* .However animal immunized with biofilm antigen with protein concentration 14 mg/ml express no mortality at 12,24 hrs post infection and only one animal died at 48 hrs. post infection.

The immunized animals died at 24 hrs post infection also showed lesions similar to those reported in the control positive group at the same period, but immunized animal with biofilm and WCS antigen showed granulomatus lesions, mononuclear cell infilteration around congested blood vessels and sinusoids as well as parenchyma of the liver with Kupffer cells proliferation in liver and moderat hyperplasia of the spleen with proliferation of lymphocytes in the periarteriolar sheath.

Death of most animals of group 1 (positive control) during 12-24 hrs.post challenge indicated that the immune system are not stimulated induced tissue damage that considered a predisposing factors for virulent *E.coli* infection. One of the immunized animal of group 4 died at 48 hrs. this result supported the presence suggestion that biofilm with high concenteration provide better protection against *E.coli* as comparative with other types of antigen in the present study,this idea was supported by low mortility in animal die during 48 hrs post infection as comparative with mortility in the other immunized group. The lymphoid tissue hyperplasia in the present study indicated immune response ,this evidence is in agreement with (21), as well as granulomatus lesion is considered as the strongest immune defence against pathogen which present till distraction of the pathogen (22).(17) expressed that the antigen stimulated immune response with variable degrees according to type of antigen and the animals that immunized with biofilm with high protein concentration 18 mg/ml express low mortality rate and this results in agreement with the present result. Very heavy bacterial isolation from internal organs of non-immunized infected group compere with moderate,mild or no bacterial isolation in other groups, may be indicated that enteropathogenic*E.coli* overcome the natural host defense mechanism,and disseminated from the site of inoculation to internal organs as aresults of high virulent factors and due to predisposing factors inducing septic shock with multiorgans dysfunction These results were agreement with (17). That showed moderate ,whole mild or no bacterial isolation were recorded in bacterial isolation from internal organs of immunized animals with WCA Ags or animals immunized with high and low protein concentration. this observation indicated that biofilm augment the immune response. This idea was in consistence with previously observation that mentioned by some authors. Immune promoters such as biofilm,whole cell sonicated antigen were generally identified as compounds that bound specifically with the cell surface receptor proteins of phagocytes or lymphocytes to stimulate the effective generation of an immune response by the cooperation of cytokines to activate the non- specific immune system of animals (23)who stated that the nature of biofilm antigen is polysaccharide and protein ,the protein is a good stimulator of cell mediated immunity , this idea is in consistence with the current study.

At 30 day post infection, the main lesion of internal organs of immunized animal are granulomatous lesions and lymphoid tissue, particularly in the biofilm antigen of immunized animal, these may be due to this type of antigen stimulated variable degrees of immune response against *E.coli* but the immune response provides partially protection

against this infection and large abscess of adhesion were recorded liver with stomach and spleen with stomach and pancreas surround by dense cellular fibrous connective tissue, this may be due to immune response stimulated the macrophage to produce cytokins that stimulated fibroblast proliferation and produce large amount of collagen fiber to limit the spread of microorganism,this idea is in agreement with (24).

The death of all of non immunized infected animal during 12-24 hrs post infection may be indicated the strain of *E.coli*used in the present study was highly virulent and overcome the host defence mechanism proliferation and dissemination to the internal organs and led to death of animals ,this idea is in agreement with (25) .(26), (17) who explained that *S.aureus* produce super antigen that led to septic shock and multi organ failar.

References:

1. **Tenaillon ,O., Skurnik, D., Picard , B., and Denamur , E.(2010)**. The population genetics of commensal *Escherichia coli*. Nat Rev Microbial **8**:207- 217
2. **Anderson GG,Goller CC, Justice S, Hultgren SJ & Seed PC(2010)**.Polysacchride capsule and sialic acid – mediated regulation promote biofilm – like intracellular bacterial communities durincystitis . Infect Immun 78(3):963-975
3. **Kaper**, **J .B ., Nataro , J. P . and Mobley, H.L.T. (2004)**. Pathogenic *Escherichia coli*. Nat. Rev. Microbial 2:123- 140.
4. **PercivalSL, Walker J, Hunter P (2000)**. Microbiological aspects of biofilms and drinking water . CRC press , New York.
5. **Shoba, F.G. and Thomas. M. (2001)**. Study of antidiarrheal activity of four medicinal plants in castor oil-induced diarrhea. J. Ethnopharmacol., 76:73-76.
6. **Guerrant, R.L.; Van Gilder, T.; Steiner, T.S.; Theilman, M.N.; Slutsker, L. and Tauxe, R.V. (2001).** Practice guidelines for the mangement of infectious diarrhea. Clin. Infect. Dis. 32: 331-35.
7. **Rodostits, O.M.; Gay, O.M.; Hincheliff, K.W. and Coststable, K.W. (2007).**Veterinary Medicine . A text book of the cattle ,horses, sheep, pigs and goats. 8th edition. Slsevier. USA. Pp:163.
8. **Sandler, R.S.; Stewart, W.F.; Liberman, J.N.; Ricci, J.A. and Zorich, N.L. (2000).** Abdominal pain, bloating and diarrhea in the United States:prevalence and impact. Dig. Dis. Sci., 45(6): 1166-71.
9. ***Schoenian, S. (2007).*** *Diarrhea (scours) in small ruminants. Maryland cooperative Exttension. Sheep and G*oat specialist Western Maryland Research and Eduction Center.
10. **Holland, R.E. (1990).** Some infectious causes of diarrhea in young farm animals. Clinical Microbiology Reviews.345-375.

11. **Brandt, L.J. and Greenwald, D. (2001).**Acute and chronic diarrhea: a primer on diagnosis and treatment. Available at: http://www.acg.gi.org/acg-dev/physicianforum/gifocus/diarrhea.html
12. **Ansaruzzaman, M.; Albert, J.; Nahar, S.; Byun, R.; Katouli, M.; Kuhn,I. and Mollby, R. (2000).** Clonal groups of enteropathogenicisolated in case control studies of diarrhea in Bangladesh .J. Med. Microbiol., 49: 177-185.
13. **Baron, E.; Peterson, L.R. and Finegold, S.M. (1994).** Bailey and Scoffs Diagnostic Microbiology. 9th ed. the C. V Mosby.,Co.USA. p.386-403.
14. **Schulze**, J.; Schiemann, M. and Sonnenborn, U. (2006). 120 years of *E. coli* Its importance in research and medicine. 6th ed. Alfred-Nissle- Gesellschaft, Germany: 11-13.
15. **Quinn**, P.J.; Markey, B.K.; Carter, M.E.; Donnelly, W.J. and Leonard, F.C. (2004). "Veterinary Microbiology and Microbial Diseases Textbook". Printed and bound in Great Britain by International Ltd. Mosby, London, Padstow-Cornwall. : 118-126.
16. **Christensen**, G .D.; Simpson, W .A.; Bisno, A. L.andBeachey, E .H. (1982). Adherence of slime–producing strains of *Staphylococcus epidermidis*to smooth surfaces. Infect Immun; 37:318–26.
17. **Yarmorad, M.A. (2013)**. Study comparative protection of biofilm produced by *Staphlococcusaureus* with other it'santigen , M .Sc .Thesis – College of Veterinary Medicine – Baghdad University .
18. **Vasudevan, P.; Nair, M.K.M.; Annamalai, T. and Venkitanarayanan, K.S. (2003).** Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet. Microbiol. 92, 179–185.
19. **Baselga, R.; Albizu, I., Amorena, B.(1994)***Staphylococcus aureus*capsule and slime as virulence factors in ruminants mastitis . A review . Vet Microbiol 39(3-4):195-204.
20. **Al-Ithawy, A. A. (2010).** Affected factors on *Pseudomonas aeruginosa* Isolated from deferent environmental sources on biofilm formation, M.Sc. Thesis submitted to the university of Anbar/college of science.
21. **Alwan, M.J. (1996).**Nocardiaosteroides studies some aspect of pathogenesis. Ph. D. Thesis-college of veterinary Miedicin-Baghdad University.
22. **Adam, D.O. (1976).** The granulomatous inflammatory response. Am. J. Path. 84: 164-191.
23. **Corbel, M. J. (1997).** Brucellosis: an overview. Emerg. Infect. Dis. 3:213-221.

24. **Ramzi, C.; Vinay, K. and Stanely, l. (1994).** Robbins pathological basis of disease. 5th edition. Philadelphia, London, Toronto, Montreal Sydney,Tokyo. PP:183-185.

25. **Dinges, M.M.; Orwin, P.M. and Schlievert, P,M.(2000).** Exotoxins of *Staphylococcus aureus*. ClinMicrobiol Rev; 13:16–34.

26. **McCormick, J.K; Yarwood, J.M; Schlievert, P.M.(2001).** Toxic shock syndrome and bacterial superantigens: an update. Annu Rev Microbiol; 55:77–104.