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BACTERIOLOGICAL AND PATHO-MOLECULAR STUDIES ON *ARCANOBACTERIUM PYOGENES* (*TRUEPERELLA PYOGENES*) CAUSING RESPIRATORYOR DIGESTIVESYMPTOMS IN CAMEL CALVES

By

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ABSTRACT

This study was carried out on 83 dromedaries camel calves aged from five to eleven month old obtained from Mattroh and Salhia farms. Samples including nasal swabs, fecal samples, heparinized blood samples and samples from internal organs including lung, liver, spleen and intestine were obtained aseptically for bacterial culture, biochemical identification, antibiogram and PCR. The bacteriological examinations revealed that *Arcanobacterium pyogenes (Trueperella pyogenes)* were the causative agent of the disease and isolated with percent of 14.45% from the whole samples. The organism was sensitive to Erythromycin, enrofloxacin, cefotaxime, enrofloxacin, tetracycline, Oxytetracyclin and doxycycline. The attained isolates were confirmed genotypically by successful amplification of the 16S rDNA gene by obtaining an expected band of 1403bp. Histopathological examination of camel calves revealed interstitial pneumonia, necrotizing enteritis and hepatic necrosis with splenic hyperplasia. The experimentally infected rabbits with isolated *Arcanobacterium pyogenes (Trueperella pyogenes*) from diseased camel calves showed similar alterations. Finally, this study concluded that *A. pyogenes* could be isolated from camel calves suffering from interstitial pneumonia and diarrhea. In addition, to reinforce the importance of knowing the profiles prior to start therapy, improveantimicrobial therapy approaches.

Keywords:

Camel calves - *Arcanobacterium pyogenes (Trueperella pyogenes)* - Bacteriological identification - Antibiogram- PCR and Histopathology.

INTRUDUCTION

Bacteria of genus *Arcanobacterium* belonging to the family *Actinomycetaceae* was first named *Actinomyces* described by **(Collins *et al.,* 1982).** Recently, based on phenotypic and moleculartechniquestheformer*Arcanobacterium pyogenes* was reclassified to *rcanobacterium pyogenes or Truperella pyogenes* **(Yassin *et al.,* 2011)**. *Arcanobacterium pyogenes* is a Gram-positive, pleomorphic bacillus. It is a common inhabitant of the upper respiratory, urogenital and gastrointestinal tracts of many domestic animals. It is a primarily animal pathogen that causes pyogenic infection in cattle **(Joist *et al*., 2005)**, dromedary camel **(Cheng *et al.,* 2010; Muna *et al*., 2017)** and farmers **(Levy *et al.,* 2009)**. The organism is usually a secondary invader via breaks in skin or mucosal integrity then enters the bloodstream and has a protease toxin and factors that prevent local inflammatory responses. It produce yellow-green purulent material of variable consistency is typical and often a foul odors to the lesions.Both the clinical course of the disease and its risk in animals are often difficult to estimate **(Reischl *et al.,* 1994 and Al-Tarazi*et al.,* 2012)**. The most frequently isolated bacterial species from clinical cases of infection in different species of farm animals are *Trueperella pyogenes*, *T. bernardiae* and *T. abortisuis* **(Yassin *et al.,* 2011, Hijazin *et al.,* 2012)**. The purulent process usually starts in the deeper layers of the lungs, gradually covering large areas of tissue with subsequent necrosis of pulmonary alveoli **(Radostitis *et al.,* 2000)**. **Kane and Diallo (2000)** isolated *Arcanobacterium pyogenes* from camel calves suffering from diarrhea and bronchopneumonia. Pneumonia outbreaks are usually observed in calves during the change from the dry to the rainy season .Vast arrays of infectious diseases that are hampering the health of adult camels also affect camel calves. Therefore, the objectives of the present work were isolation and identification of *Arcanobacterium pyogenes* from camel calves showing digestive and respiratory manifestations. The present work was carried out in farms of some Egyptian governorates through applying the Antibiogram on isolated organism and by using PCR to confirm the isolate. Also the histopathological examination of affected organs and experimentally infected laboratory animals with the isolated bacterium to compare the alterations in camel calves by lab animal inoculation for insuring the diseases caused by the isolated organism.

MATERIAL AND METHODS

1-Bacteriological examination.

Animals:

A total number of 83 camel calves aged from five to eleven month old obtained from Mattroh and Salhia farms were employed for the study.

Sampling:

Nasal, fecal swabs and hepranized whole blood samples were collected from 83 dromedary camel calves of age five to eleven month old from the diseased and apparent healthy calves. One hundred tissue specimens (lung, liver, spleen and intestine) were obtained from 25 animal (I0 slaughtered and 15 recently dead).Detailed data are shown in (Table 1).Visual, palpate and incise method was carried according to **(Taiwo, 2005)**.

Table (1): Number and types of samples collected from camel calves.

Animal status		No. of animals	Type and No. of Samples				Total of samples
			Whole blood	Nasal and fecal swab		Tissue samples	
				N.S	F.S		
Suspected cases	Clinically sick	53	53	53	53	-	159
	recently dead calf-camel	15	-	-	-	60	60
	Slaughtered calf camel	10	-	-	-	40	40
Apparent healthy		5	5	-	-	-	5
Total numbers		83	58	53	53	100	264

Cultivation:

All nasal ,fecal , whole blood samples and internal organ samples were separated into 2 parts, First one was inoculated directly into nutrient broth for 24 hours at 37ºC then sub cultured onto Mac Conkey's agar , blood agar .The inoculated plates were incubated at 37ºC for 24-48 hours for isolation of *Arcanobacterium pyogenes*. Heparinized whole blood samples were directly cultured onto Blood agar, incubated at 37ºC for 24 hours and examined microscopically after applied Gram staining technique.The isolation, purification, biochemical Identification of the bacterial isolates were carried out according to **koneman *et al.*, 1997.**

Isolation and identification of bacteria:

Swabs from the lungs, liver, spleen and intestine were streaked on plates containing blood agar enriched with 5 - 6% sheep blood and incubated at 37°C for 24 h with further re-incubation for 36 - 72 h, if no growth was observed after 24 h. Single colonies of different types were picked on plates containing blood and on MacConkey's agar. The pure cultures were Gram-stained. Identification of bacterial agents was through cultural, morphological and in some cases biochemical characteristics **Confer, (2009)**. Positive Samples for bacteria other than A. *pyogenes* were excluded. The diagnosis was later confirmed using the API CORYNE 20 test (Biome'rieux, Marcy 'Etoile, France).

Antibiogram pattern:

Antibiogram was applied on the different isolated samples using in vitro disc diffusion technique according to **(Quinn *et al.,* 2002).** It was performed on Mueller Hinton agar plates and 12Discsofchemotherapeuticagents. The used antibiotics included Erythromycin E(15µg), Trimethoprim/sulphamethazole SXT (25 µg), Norfloxacin Nor (10 µg),Cefotaxime CTX (30µg),Mecillinam MEC (10µg), Enrofloxacin ENR(5µg), Lincomycin L (2µg), Doxycycline Do (30 µg), Oxacillin OX (1 µg),Tetracyclin TE (30 µg),Oxytetracyclin T (30 µg),and Ampicillin AM (10 µg). The results were interpreted according to **EUCAST (2016).**

B-PCR assay:

1-DNA extraction: A boiling procedure was used to extract DNA from bacterial isolates according to **Reischl *et al.* (1994).**

2- PCR assay targeting 16S rDNA gene of Arcanobacterium pyogenes was performed. By using total volume of 25ul reaction mix, contain 5ul of template DNA, 20 pmol of each primer and 1X of PCR mix (PCR Master Mix, Fermentas, and Life Science). The PCR cycles were carried out in Eppendorf AG (22331 Hamburg) thermocycler. Detailed sequences of primers and cycling protocols are depicted in (Tables 2, 3). The analysis of PCR products were carried out using 1.5% ethidium bromide stained agarose gel.

Table (2): Primers used in PCR assays.

Target	Name (strand)	Primer sequence (5 - 3)	Reference
A. *pyogenes*	**16S rDNA UNI-F**	**5′-AGAGTTTGATCATGGCTCAG-3′**	**Kuhnert**
	16S rDNA UNI-R	**5′-GTGTGACGGGCGGTGTGTAC-3′**	***et al.* (1996)**

Table (3): Cycling protocols of PCR assay.

Target	Amplicon size	Cycling program			
		Step	Temp.	Time	No. of cycles
16S rDNA gene	1403bp	Initial denaturation	95ºC	10 min	One cycle
		Denaturation	95ºC	45s	30 cycles
		Anealing	58ºC	45s	
		Extention	72ºC	1min	
		Final extention	72ºC	7min	One cycles

3-Experimental Design:

Six New Zealand White rabbits at age of 3months old and of (1-1.1/2kg b.wt.) were obtained from farm of Animal Reproduction Research Institute (ARRI) used for the experimental infection with *A. pyogenes* isolate from the affected lungs, liver, spleen and intestine of the camel calves. Rabbits were divided into 2 groups: The first group (4 rabbits) was inoculated with 0.5 ml of the isolated strain of *Arcanobacterium pyogenes* by I / P and per nose. The second group was kept as control according to **(Archibald and Chen, 1985)**.

Pathological examination:

I00 tissue specimens were taken from 25 camel calves, 10 affected animals immediately after slaughtering and 15 animals which recently dead in the farms.The affected lungs, liver, spleen and intestine were collected after carful gross examination then divided into two portions. One was fixed in 10 % formal saline for histopathological examination and the other one was placed in sterile plastic bags and kept in an icebox to be subjected to bacteriological examinations later.Fixed tissuespecimens of the lung, liver,spleen and intestine of the infected camel calves also, the lung, liver, spleen and intestine of the experimentally infected lab. Rabbits with *A.pyogenes* isolated from tissues of camel calves were dehydrated in different grades of alcohol, then cleared by xylol,embedded in paraffin, sectioned at 3u thickness and finally stained with hematoxylin and eosin according to **Bancroft and Stevens, (1990)**.

RESULTS

1-Bacteriological results:

Twelve *Arcanobacterium pyogene* isolates representing percentage of 14.45% were recovered from 83 camel calves. The highest incidence from lung was four isolates (4.81%) followed by liver and intestine three isolates (3.61%) from each then form spleen (2) isolates (2.40%). Antibiogram pattern of *A. pyogenes* recovered from diseased camel calves were recorded in (Table 4), Fig. (17).

Table (4): In vitro sensitivity test of *Trueperella pyogenes* recovered from diseased camel calves.

Chemotherapeutic agents	%
Erythromycin E (15µg)	54
Trimethoprim/sulphamethazole SXT (25 µg)	0
Norfloxacin Nor (10 µg)	88
Cefotaxime CTX (30 µg)	74
Mecillinam MEC (10 µg)	0
Enrofloxacin ENR (5 µg)	100
Lincomycin L (2 µg)	0
Doxycycline Do (30 µg)	100
Oxacillin OX (1 µg)	0
Tetracyclin TE(30 µg)	92
Oxytetracyclin T(30 µg)	78
Ampicillin AM (10 µg)	18

2-PCR results:

The isolates were confirmed by PCR assay targeting 16S rDNA gene of A. pyogenes and successful amplification of predicted 1403bp was obtained Fig. (18).

3. Clinical signs and pathological results:

3-A. Clinical signs:

Clinical manifestations appeared on the camel calves is fever (41.5ºC), anorexia, intermittent cough with mucous discharge from eyes and nose, distress, diarrhea then occasional death of 10 calves in Mattroh farms and 5 calves in Salhia farms were observed. All dead calves were nearly five to eight month of age.

3-B. Pathological examination:

1-Lung of camel calf: Macroscopical examination of infected camel calf showed reddish discoloration of some surface areas of lung with paleness of other Fig. (1).

Lung of inoculated rabbit with *A. pyogenes* showed hyperemia and consolidation of the diaphragmatic lobes of the lung Fig. (2).

Microscopic examination of lung of infected camel calf revealed destruction and desquamation of the lining epithelium of the bronchi and focal accumulation of the inflammatory cells mainly lymphocytes and neutrophils with increased the interalveolar septa. Emphysema and moderate interstitial hemorrhages were noticed Fig. (3).

Lung of inoculated rabbit showed emphysema and edema of the interstitial tissue. Diffuse areas of interstitial hemorrhages and inflammatory cell aggregations mainly lymphocytes and some neutrophils Fig. (4).

2-Macroscopical examination of spleen of infected camel calf showed congestion with morbid and edematous organ Fig. (5).

Spleen of inoculated rabbit showed edematous and congested organ with fatty elongated part Fig. (6).

Microscopic examination of spleen of camel calves revealed hyperplasia of lymphoid follicles with marked congestion Fig. (7).

Spleen of inoculated rabbit revealed spleen hyperplasia (increase in the number of splenic follicles) with necrosis of some lymphoid follicles Fig. (8).

3-Liver: Macroscopically: the liver of infected camel calf showed edematous, congested and hyperemic organ. Whitish focal area appeared on the surface of the liver Fig. (9).

The liver of inoculated rabbit showed congested and hyperemic organ with whitish focal area appeared on the surface of the liver Fig. (10).

Microscopic examination:

The liver of infected camel calf revealed with hyperplasia of the bile duct epithelium with lymphocyticinfiltration.Degeneration of hepatocytes and periductular fibroplastic proliferation was noticed Fig. (11).

Liver of inoculated rabbit revealed degeneration of hepatocytes and necrosis of others. Focal lymphocytic aggregation with some neutrophils and activation of von kupffer cells Fig. (12).

4- Intestine: Macroscopically:

Intestine of infected camel calf showed edematous organ with hyperemic dots appeared on the surface of the organ Fig. (13).

Intestine of inoculated rabbit showed edema of some parts with moderate congestion of the organ Fig. (14).

Microscopic examination:

Intestine of infected camel calf showed necrotizing enteritis characterized by degeneration with desquamation of the epithelial cells of the intestinal villi, which infiltrated with mononuclear inflammatory cells mainly lymphocytes, neutrophils and some macrophages. Congestion of blood vessels was shown in Fig. (15).

Intestine of inoculated rabbit showed degeneration and necrosis of the intestinal glands with desquamation of the epithelial cells of the intestinal villi and marked aggregation of the inflammatory cells mainly lymphocytes, neutrophils and some macrophages Fig. (16).

DISCUSION

Arcanobacterium pyogenes(A. pyogenes) is a gram-positive, irregular, non-motile, non-spore forming bacterium that is one of the important opportunistic pathogens of the upper respiratory, urogenital and gastrointestinal mucosa of animals **(Ghadrdan and Yosefi, 2004; Ertaş *et al.*, 2005 and Joist and Billington 2005)**. Bacteriological examination in our study revealed 12 *A. pyogene* isolates from the collected tissue samples with a percentage of 14.45% and highest incidence was in the lung and liver. **Walaa and Amal (2017)** mentioned similar findings**.** They isolated Arcanobacterium *pyogenes (A. pyogenes)* from lung and liver of slaughtered cattle. It presented 16.04% in Mansoura abattoir and Damietta local dairy farms. In addition, **Muna *et al.* 2017** isolated *A. pyogenes* by percent of (11.25%) from lung of camel with inerstitial pneumonia in Sudan. Our results come nearly to these ratios but come higher than that of **Al-Tarazi (2001)** who isolated the bacterium by percent of (6.66%) from pneumonic lung of dromedary camels in Jordan. This high prevalence rate could be attributed to environmental changes and husbandry practices, mixed herding and sharing of water and pasture with adult animals and young calves.

This suggested that, the immune system of young animals is weaker than those adults, which make young animals more vulnerable to infection with pyogenic microorganisms **(Dubai *et al.*, 2004; Kaufmann, 2005 and Bekele, 2014)**. The interpretation of antimicrobial

susceptibility testing results for *A. pyogenes* and confirmation of the results with published data may be difficult where the divergent results of *A. pyogenes* antimicrobial susceptibility testing were reported for β-lactam antibiotics **(EUCAST, 2016 and Zhao *et al.,* 2011)**. In the present study all, the strains were susceptible to β-lactams regardless of strain origin. Antibiogram results revealed 100% resistance to trimethoprim /sulphamethazole, Mecillinam, Oxacillin and Lincomycin. In contrast, the results showed 100 % sensitivity to Enrofloxacin and Doxycycline. While the sensitivity showed different percentage to Tetracyclin 92 %, Norfloxacin 88%, Oxytetracyclin 78%, Cefotaxime 74%, Erythromycin 54% and Ampicillin 18%. Additionally, 16S rDNA gene gives more species that are precise identification of clinical bacteria of veterinary origin. Furthermore, **(Zhao *et al.,* 2013)** had concluded that 16S rDNA is among the most informative targets and considered the most fundamental molecular approach to ascertain different microorganisms.Respiratory disorders are still serious problem facing camel rearing. The importance of respiratory diseases of camel comes from their prevalence, effect on productivity and for some extent, their international spread **(Abass and Omer, 2005)**.Histopathological examination of lungs of camel calves revealed thickening of the alveolar wall and interlobular septa, infiltration of inflammatory cells, emphysema, necrosis and sloughing of bronchiolar lining epithelium. These results come in agreement with those of **(Muna *et al.,* 2017)** in Sudan. In the present study, pathological examination of lungs of camel calves revealed different pulmonary lesions, the lesions associated with changes in air contents such as emphysema or inflammatory lesions as interstitial pneumonia and bronchitis also, hemorrhages and edema. The incidences of lung lesions are nearly similar to those recorded by **(Al-Tarazi, 2001**) who recorded the same lung lesions among slaughtered camels in Northern Jordan and **(El-mahdy *et al,* 2013)** in Saudi Arabia. Histopathological sections of liver showed degeneration and necrosis of hepatocytes with infiltration of inflammatory cells and proliferation of bile ducts signed chronic infection. Similarly **(Nourani and Salimi, 2013)** noted the abundance of hepatocellular degeneration, necrosis associated with toxic hepatic lesions of the dromedary camel. Importantly, the isolation of *Arcanobacterium pyogenes* in this study strongly suggested its involvement with liver necrosis;these investigations were similar to those of **Zuhair (2017)**. Such organism enters the blood stream then disseminates to cause septic arthritis and sometimes abscesses in various organs and tissues, but mainly in the lungs and liver **(Dubai *et al.,* 2004 and Taha**

***et al.,* 2007)**. Spleen hyperemia is common in septicemias as spleen is an important part of immune system to clear the blood from bacteria with its red pulp macrophages. Reactivity of white pulp is related to lymphocyte activation and mitoses show the antigenic irritation due to bacteria or any other sources **(Fry and Gavin, 2007)**. The histopathological alterations of the affected spleen, liver and lung of camel calves come in agreement with the results recorded by **(Hermida *et al.* 2004; Kebede *et al.,* 2010 and Aljameel *et al.,* 2014).** In this study, degenerative changes were observed in the affected organs of the animals with marked necrosis of hepatic cells and spleen hyperplasia. These investigations were similar to that of **Tehrani *et al.,* 2012** in liver of adult camels where the disease progressed by chronisity to liver abscess. The desquamation of the intestinal lining epithelium of small intestine indicated the toxic effect of *Arcanobacterium pyogenes* and the role of its toxins in the disease process. However, upon exposures to the toxins, the villas tips enterocytes became degenerated and were sloughed into intestinal lumen leaving denuded basement membranes, which allowed fluid leakage and attract leucocytes into the lamina propria causing necrotizing enteritis. These results come in agreement with the results reported by **Jarosz *et al.,* (2014)** in small animals and come in parallel to that reported by **Zaki *et al.,* (2000)** in buffalo and in adult camels.

CONCLUSION

The present study serves to highlight the fact that *Arcanobacterium pyogenes* can be isolated from calf camel suffering from digestive and respiratory manifestation with predominance of interstitial pneumonia, necrotizing enteritis and hepatic necrosis. It also reinforces the importance of knowing the susceptibility profile before initiating therapy, to improve antimicrobial therapy approaches.

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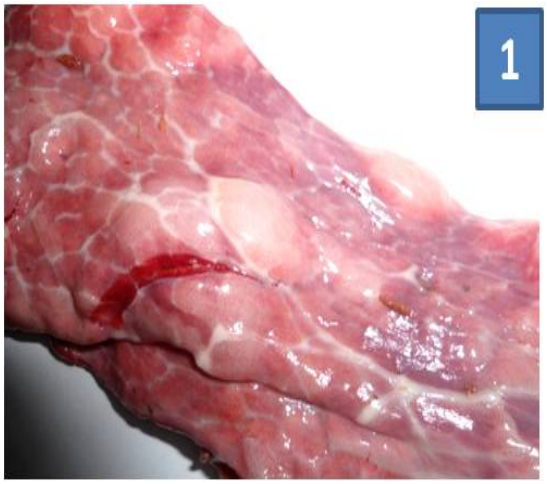
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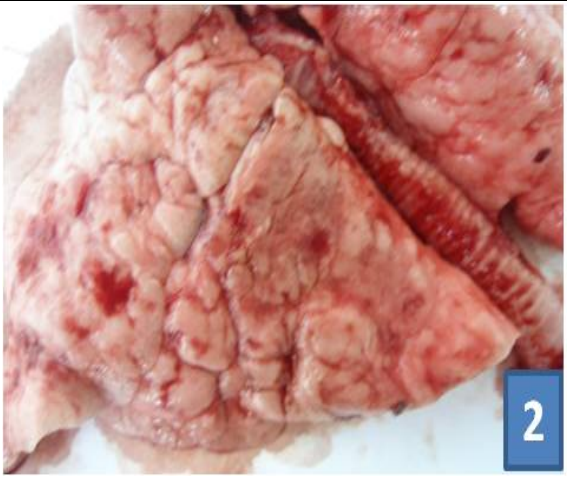
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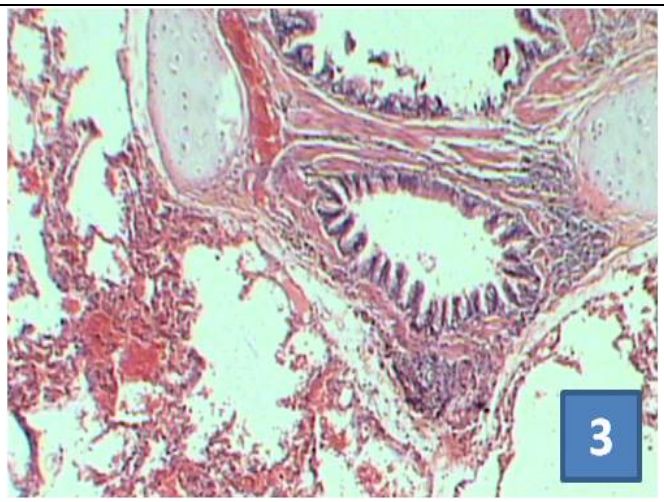
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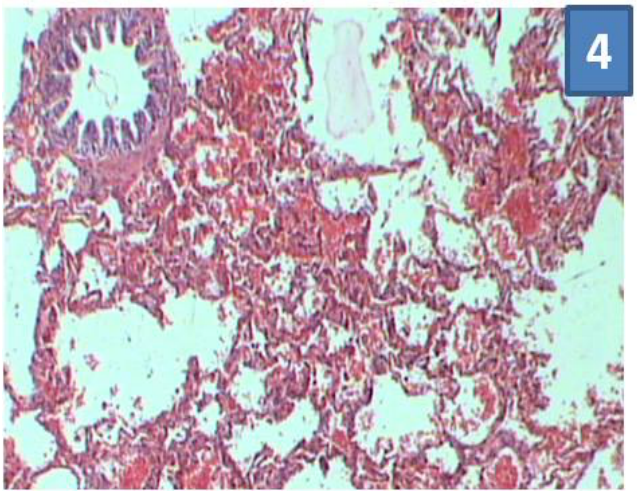
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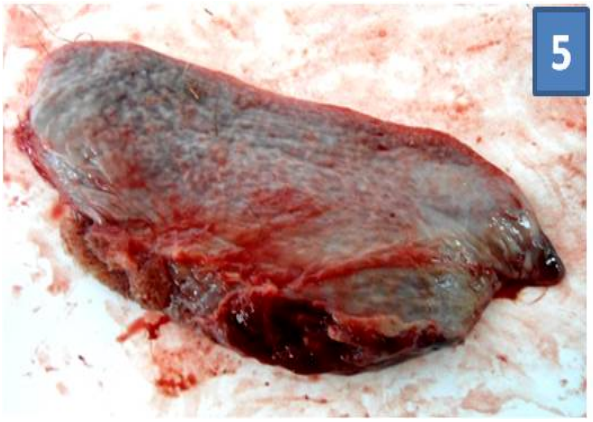
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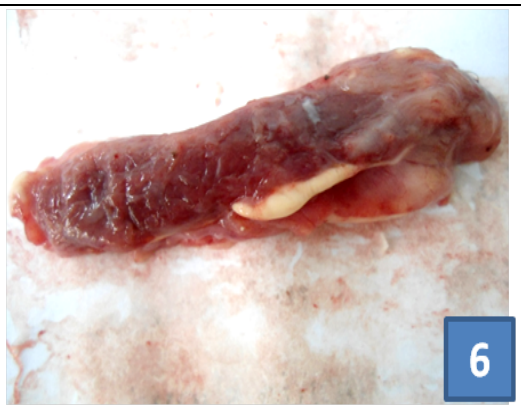
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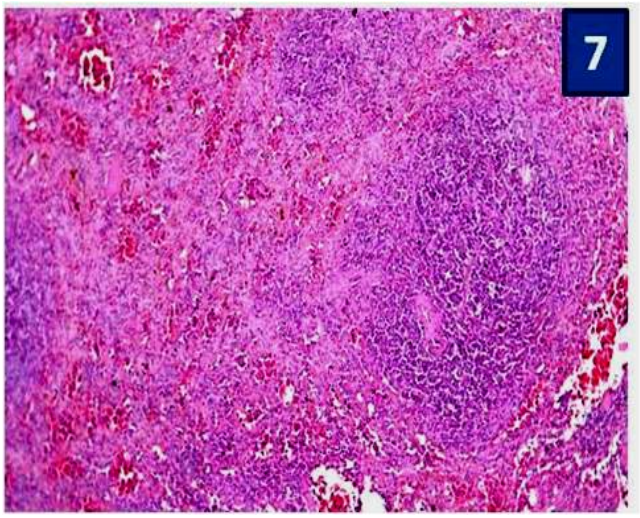
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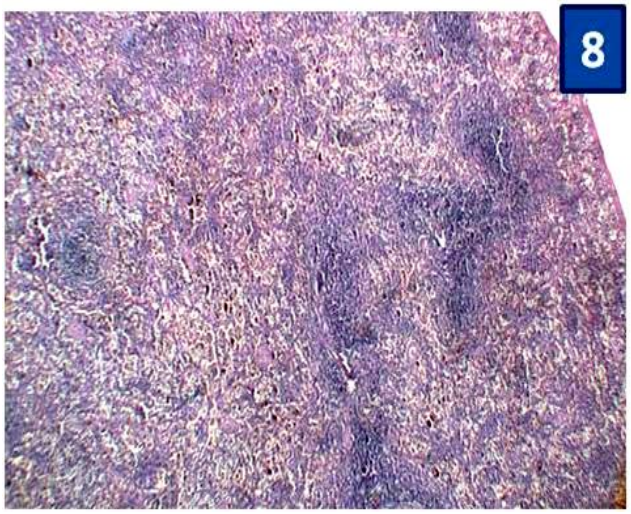
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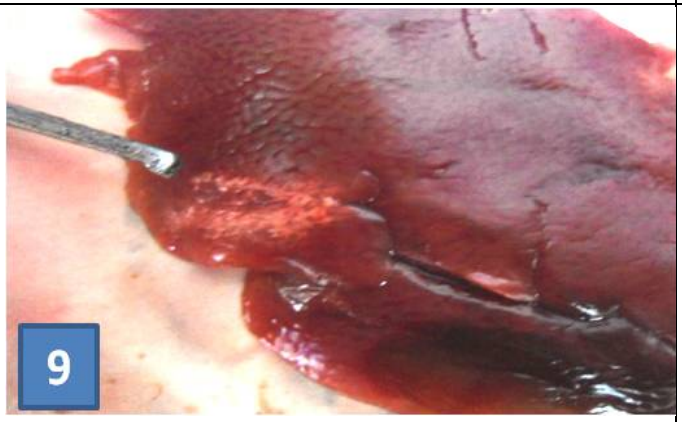
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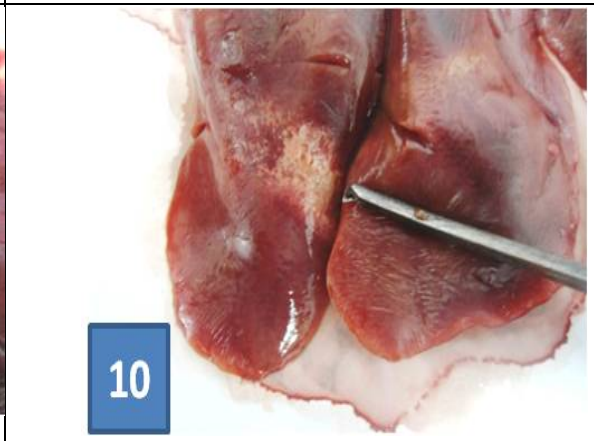
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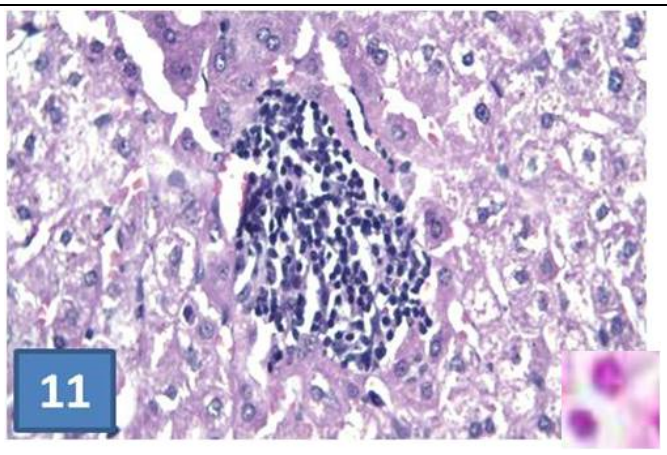
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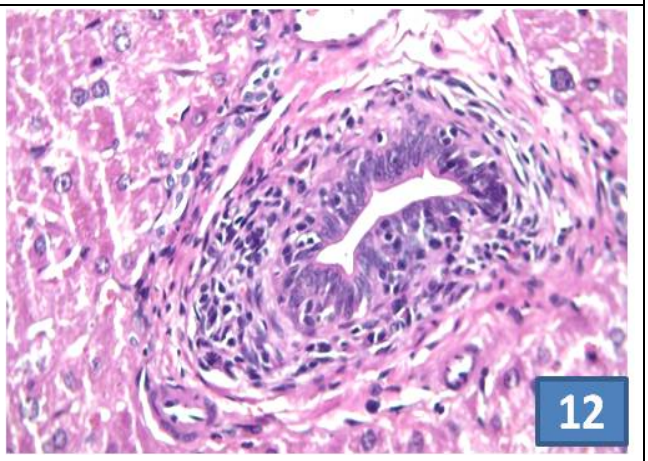
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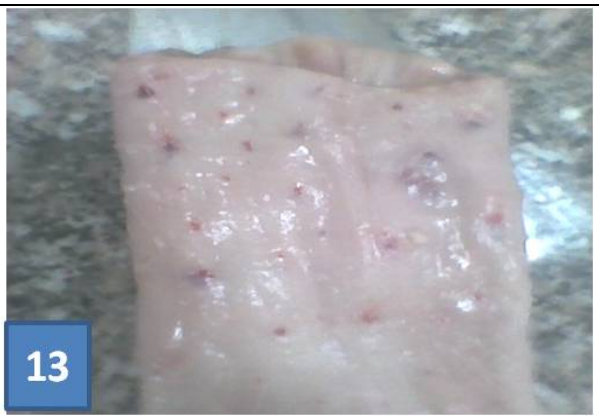

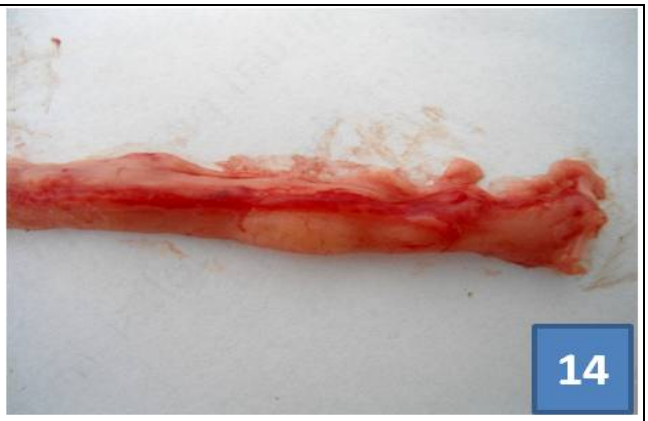

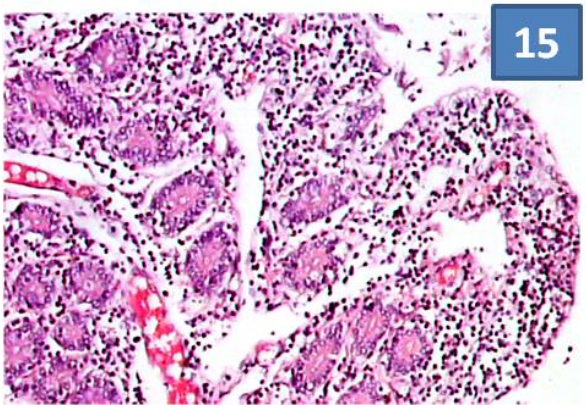

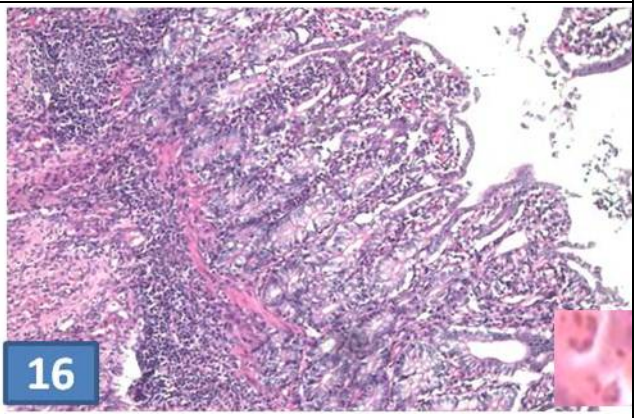

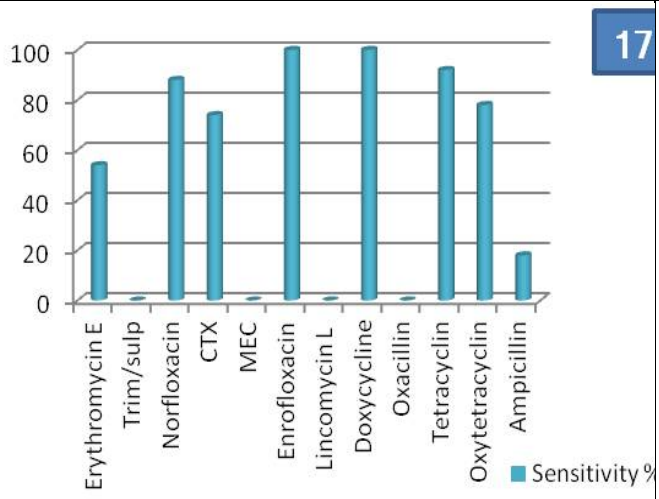

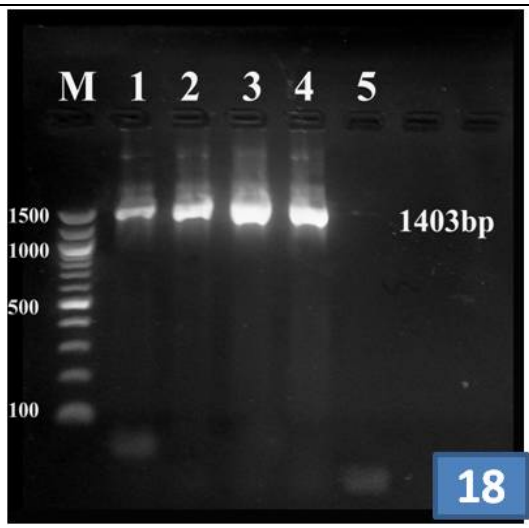

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Fig. (1): Lung of camel calf infected with *A .pyogenes* showed reddish discoloration of some areas of the lung with paleness of other.

Fig. (2): Lung of rabbit inoculated with *A .pyogenes* showed hyperemia and consolidation of the diaphragmatic lobes of the lung.

Fig. (3): Lung of camel calf infected with *A. pyogenes* showed destruction and desquamation of the lining epithelium of the bronchi with focal aggregation of the inflammatory cells mainly lymphocytes and neutrophils. Moderate interstitial hemorrhages with thickening of the inter alveolar septa and emphysema were noticed. **H&E. X100.**

Fig. (4): Lung of rabbit inoculated with *A. pyogenes* showed emphysema and edema of the interstitial tissue. Diffuse areas of interstitial hemorrhages and inflammatory cell aggregations mainly lymphocytes and some neutrophils. **H&E. X100.**

Fig. (5): Spleen of camel calf infected with *A .pyogenes* showed congestion with morbid and edematous organ.

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Fig. (8): Spleen of rabbit inoculated with *A. pyogenes* showed increasing in the number of hyperplasic follicles with necrosis of some lymphoid follicles.**H&E.X100**.

Fig.(9): Liver of camel calf infected with *A. pyogenes* showed edematous, congested and hyperemic organ with whitish focal area appeared on the surface of the liver.

Fig. (10): Liver of inoculated rabbit with *A. pyogenes* showed edematous, congested and hyperemic organ. Whitish focal area appeared on the surface of the liver.

Fig. (11): Liver of camel calf infected with *A. pyogenes* showed hyperplasia of bile duct epithelium with lymphocytic infiltration and neutrophils. Degeneration of hepatocytes and periductular fibroblastic proliferation was noticed. **H&E. X400.**

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Fig.(13): Intestine of camel calf infected with *A. pyogenes* showed edematous organ with dots of congested petechea of heamorrhages appeared on the surface of the organ.

Fig.(14): Intestine of inoculated rabbit with *A .pyogenes* showed edema of some parts with moderate congestion of the organ.

Fig. (15): Intestine of camel calf infected with *A. pyogenes* showed degeneration and dilatation with desquamation of the epithelial cells of the intestinal villas which infiltrated with mononuclear inflammatory cells mainly lymphocytes, neutrophils and some macrophages. Congestion of blood vessels was showed.**H&E.X100.**

Fig.(16): Intestine of rabbit inoculated with *A. pyogenes* showed degeneration and necrosis of the intestinal glands with desquamation of the epithelial cells of the intestinal villas and marked aggregation ofthe inflammatory cells mainly lymphocytes, neutrophiles and some macrophages**.H&E.X100.**

Fig. (17): Chart of sensitivity test of *Arcanobacterium pyogenes* recovered from diseased camel calves.

Fig. (18): Ethidium bromide stained a garose gel electrophoresis of PCR assay Lane M: 100bp plus DNA ladder Lane 1: Positive control, Lanes 2-4: positive isolates with expected band of 1403bp, Lane 5: Negative control.