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Bacteriological and Molecular studies on *C. pseudotuberculosis equi* Causing oedematus skin disease in Buffaloes

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Abstract

Oedematus skin disease (OSD) among buffaloes is an acute disease manifested by superficial swelling which culminate in the formation of abscesses and release of serous bloody exudates. Fifty aspirates collected from closed swellings of buffaloes hosted and owned sporadically in different Egypt governorates mainly in hot weather showing clinical symptoms of the OSD were examined bacteriologic ally for prevalence of *C. pseudotuberculosis equi*, polymerase chain reaction (PCR) for detection of virulence genes of the isolates. A total of(26) isolates were identified as *C. pseudotuberculosis equi* in a percentage of (52%), other isolated bacterial species included Staphylococcus species and streptococcus species either single or mixed infection. All C. *pseudotuberculosis equi* isolated exhibited a synergistic hemolytic activity with *Rhodococcus equi* culture filtrate (Modified CAMP test) and inhibited staphylococcal hemolytic activity (Reverse CAMP test). Isolates were tested for antimicrobial susceptibility by disc diffusion method against group of antimicrobial agents of the most common used in the fields. The test revealed that, most of the isolates were sensitive toamoxicillin/clavulanic acid, and trimethoprim/ sulphmethazole followed by enerofloxacin and erythromycin. On the other hand the tested isolates are highly resistant to ampicillin, and nalidixic acid. Application of polymerase chain reaction (PCR) was done on a random six *C. pseudotuberculosis equi* isolates, which revealed that, all 6 tested isolates were positive for presence of *pld*, *rpoB* and *sigE* genes, except one isolate negative for *sigE*. These results proved that, *pld, rpoB* and *sigE* genes has been implicated as the major virulence factors of *C. pseudotuberculosis equi* and also incriminated as the main player of pathogenesis of OSD in buffaloes leading to the acute form of the disease and their severity of virulence depends upon the number of factors in the same strain which may explain the variety in clinical picture of the disease.

Key word: *Buffaloes, oedematous skin disease, C. pseudotuberculosis equi, virulence genes.*

Introduction

Corynebacterium pseudotuberculosis is a causative organism of acute disease in buffalo known as oedematous skin disease (OSD) and caseous lymphadenitis (CLA) in sheep. Human affected with the disease show liver abscess and abscess in the internal lymph nodes **(Moussa *et al.*, 2016).** This microorganism is a facultative intracellular pathogen that exhibits pleomorphic forms, such as coccoids' and filamentous rods, it is a non-sporulation, non-capsulated and non-motile bacterium; however, this bacterium is a facultative anaerobe and grows best at 37°C, at a pH of 7.0 to 7.2. It grows sparse initially on the agar surface and then becomes organized in clumps or in palisades, taking on a cream to orange coloration; colonies are dry, opaque and concentrically ringed **(Dorella *et al.*, 2006)**.

In Egypt, buffalo OSD is an endemic disease caused by *C. pseudotuberculosis* biovar II

named *C. pseudotuberculosis equi* which is toxigenic producing diphtheria toxin characterized mainly by reduction nitrates to nitrites by the action of nitrate reeducates gene (*narG*) **(Soars *et al.* 2013; Selim *et al.,* 2016; Oliviera *et al*., 2016** and **Almeida *et al.,* 2017)**. It appears as outbreaks during the summer months especially in lower Egypt which is characterized by high humidity and usually associated with the breeding season of the blood sucking fly *Hippobosca equina;* the main transmitter of the causative agent. The disease is initiated by intradermal inoculation of *C. pseudotuberculosis equi* in buffalo skin through biting of the blood-sucking insects especially *H. equine* fly **(Sohier *et al.,* 2008**; **Moussa *et al.,* 2016** and**Viana *et al*., 2017)**.

Many studies on open or ruptured lesions suggested that transmission may be mechanically with contaminated environment (soil & water) with *C. pseudotuberculosis* mainly by Musca Domestica **(Sayed, 2001 and Spier *et al.,* 2004)**. Although mortality is low, morbidity is high and the treatment of diseased animals may extend for months causing economic losses to farmers due to reduction of animal productivity; decrease in work efficiency of the animal, expensive medicaments and surgical intervention in some cases which represent a noxious situation for owners and veterinarians **(Selim, 2001)**. It is well documented that *C. pseudo tuberculosis equi* (nitrate positive), exerts its pathogenesis by secretion of exotoxin (s) including phospholipase D (PLD) which has been implicated as the major virulence factor and incriminated as main player of pathogenesis of OSD **(Baird and Fontaine, 2007)**.

In spite of both biotype 1 (nitrate negative) the causative agent of caseous lymphadenitis (CLA) in sheep and goat and biotype 2 (nitrate positive) produce PLD, there is a difference in pathogenicity for guinea pigs, whereas, biotype 2 shows more rapid and reverse hemorrhagic lesions at site of inoculation associated with rapid death of the experimental animals and this may be attributed to the presence of toxigenic factor (s) beside PLD **(Guimarães *et al*., 2011)**. Phospholipase D (PLD) gene protects the bacteria from killing by phagocytic cells and enables bacteria to escape from neutrophils and impair neutrophils chemo taxis toward the site of infection **(Hodgson *et al.,* 1994**). PLD gene increase vascular permeability and bacterial survival in the host **(Dorella *et al.,* 2006)**. The microorganism continues to multiply in host cells after being taken up by macrophages, which are disrupted and the microorganism is released, microorganisms are subsequently taken up by other circulating phagocytic cells and the cycle is repeated. This repeated phagocytosis cycle has been reported to cause recurrent lesions in *C. pseudotuberculosis* infections **(Yeruham *et al.*, 1997)**.

Nabih *et al.* (2018) isolated and identified *C. pseudotuberculosis* which was examined for evidence of virulence genes Phospholipase D (*pld*) and β-subunit of RNA polymerase (*rpoB*) by polymerase chain reaction (PCR), and they found that: *Pld* gene-based PCR is more reliable than *rpoB* gene based ones for the diagnosis of *C. pseudotuberculosis.* There is a lack of information about the virulence factors of *C. pseudotuberculosis* and the pathogenic mechanisms of the diseases other than CLA caused by this bacterium (**McKean *et al.*, 2005)**. The observations of severe toxic activity of buffalo strains may be attributed to the difference of antigenic structures of both biotypes and may be consequently that there are other virulence factors in addition to PLD toxin and one of the predicted toxins is the diphtheria toxin (DT) (tox) which may be produced by sporadic isolates of biotype1 and closely related type of Corynebacterium known as *Corynebacterium ulceranc* **(Eva *et al*., 2013)**.

The present study aimed to throw the light on oedematous skin disease which affects many buffaloes in Egypt and its causative agent *C. pseudotuberculosis* serotype II (*C. pseudotuberculosis equi*) as well as detection of the virulence genes of the isolates by PCR.

Materials and Methods

Samples: Fifty aspirates were collected from closed oedematus skin lesions of water buffaloes hosted and owned sporadically from different Egypt governorates mainly in hot weather showing developed swellings which usually appear in a diffuse form with thickening of the skin in the hind or fore limbs, the belly and the dewlap. After disinfecting the skin surface using 5% tincture iodine, the aspirate were separately collected in sterile properly capped labeled bottles and taken in a cold container to the laboratory for bacteriological examinations.

Isolation and identification of causative bacterial agents: Each aspirate sample was cultured directly onto brain heart infusion agar plates and sheep blood agar plates then was incubated at 37°C for 24-48 hours aerobically. The growing surface colonies were identified by cultural, morphological and biochemical characters according to **(Quinn *et al.,* 2005 and Anon, 2007)**.

Biochemical identification of *C. Pseudotuberculosis* biotype 2: Colonies identified as *C. pseudotuberculosis* according to cultural and morphological examination were subjected to the biochemical tests as prescribed by **(Quinn *et al.,* 2011)**. Catalase, urease and nitrate reduction positive which is specific reaction which differentiate the biotype 2 (buffalo origin) from biotype1 (sheep origin). The bacteria ferment glucose and fructose but not ferment galactose, sucrose, lactose, mannitol, or xylose. The identification of *C. pseudotuberculosis* was based on the results of both standard biochemical tests **(Efstratiou and George 1999** and **Funke and Bernard, 2007)** and a commercial kit: the API Coryne System (bioMérieux, LaBalme-les-Grottes, France).

Detection of hemolytic Activity by Modified and Reverse CAMP test: Synergistic haemolysis with *Rhodococcus equi (R. equi)* and inhibition of beta haemolysis of with *S. aureus* (*R. equi* and *S. aureus* isolated in a previous study) were examined. The obtained *C. pseudotuberculosis* isolates were screened for their PLD activity by detection of their synergistic hemolysis with *R. equi* filtrate using sheep erythrocytes by hemolytic assay of *C. pseudotuberculosis,* "Modified CAMP" **(Songer *et al.,* 1990)**. Moreover they were screened for the antagonistic hemolysis with *S. aureus* "Reverse CAMP**" (Egen *et al.,* 1989)**.

Antibiogram sensitivity test: Isolates were tested for antimicrobial susceptibility on Mueller Hinton agar by disc diffusion method according to the Clinical Laboratory Standards Institute **(CLSI, 2012)**. The following antibiotic disks were used: amoxicillin/clavulanic acid (AMC 30 µg), oxytetracycline (T 30 µg), streptomycin (S 10 µg), trimethoprim (TM 2.5µg), cloxacillin (CX 5µg), gentamicin (CN 10) and penicillin (P 10IU), Tetracycline (TE 25µg), Kanamycin (K15µg) and Erythromycin (15µg). The plates were incubated at 35°C for 24 hours. The zone of inhibition around each disc was measured and the interpretation was made as per the zone size interpretation chart provided CLSI by the disc manufacturer.

Detection of virulence genes of isolates by PCR:

DNA extraction: DNA extraction from random six isolates of C. *pseudotuberculo*sis was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer: Primers used were supplied from Metabion **(Germany)** are listed in Table (1).

PCR amplification: Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied bios stem 2720 thermal cycler the cycles of each primer were illustrated in Table (1).

Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A gel pilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) and gene ruler 100 bp ladder (Fermentas, Thermo-scientific) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (AlphaInnotech, Biometra) and the data was analyzed through computer softwar

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers Sequences	Amplifie segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Pld	ATA AGC GTA AGC AGG GAG CA	203	94˚C 5 min.	94˚C 15 sec.	56˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	**Ilhan *et al*., (2013)**
	ATC AGC GGT GAT TGT CTT CCA GG							
RpoB	CGWATG AACATY GGBCAG GT	406	94˚C 5 min.	94˚C 15 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Sammra *et al*., (2014)**
	TCCATY-TCRCCRA ARCGCT G							
SigE	GGMAC-CGCAGC DTTCGA CGC	490	94˚C 5 min.	94˚C 15 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Pacheco *et al.*, (2012)**
	CGTCCRC (GGT-GRATWC GGGA							

Results and Discussions

Oedematous skin disease (OSD) appears mainly among buffaloes and occasionally cattle in Egypt confined to Lower Egypt as a result of the suitable climatic conditions **(Selim, 2001; Mohamed** and **Reda, 2015)**, especially during late spring and early summer correlating with *Hippobosca equina* breeding season and lesions are associated with its predilection seats of infestation (hairless areas as axilla and groin, inner aspect of limbs and under tail) **(Syame *et al*., 2008** and **Sokół and Michalski, 2015)**.

The disease is characterized by consistent signs of OSD with developed swellings which usually appear in a diffuse form with thickening of the skin in the hind or fore limbs, the belly and the dewlap. These lesions usually involve drainage lymph nodes, which may be enlarged and attain the size of a small watermelon. This picture of the disease is different from that occurs in sheep (chronic lymphadenitis) caused by the *C. pseudotuberculosis ovis (*biotype1) **(Selim, 2001)**.

The illustrated results in Table (2)showed that, *C. pseudotuberculosis equi* was isolated as single and mixed infection from 26 samples in a prevalence rate of 52%. Other bacterial species isolated from the examined OSD samples included *staphylococcus* spp. 17 isolates in prevalence rate of 34% and *streptococcus* spp., 16 isolates in prevalence rate of 32%. On the other hand 5 samples were negative for bacterial isolation. Mixed infection of all recorded three organisms was noticed in 14 samples.

Table (2). Bacterial species isolated from fifty examined samples:

Bacterial species	No.	%
Single infection:		
C. pseudotuberculosis equi	21	42
***Staphylococcus* species**	5	10
***Streptococcus* species**	5	10
Mixed infection:		
***C. pseudotuberculosis equi*+ staphylococci.**	3	6
***C. pseudotuberculosis equi*+Streptococci**	2	4
Staphylococci +Streptococci.	9	18
-Ve bacterial isolation:	5	10

These results revealed that, the main or primary cause of the oedematus skin disease (OSD) in buffaloes were *C. Pseudotuberculosis equi* strain and may be followed by contamination with other bacteria as *staphylococcus* and *streptococcus* species which considered one of the most important causes of the skin diseases so lead to complication in the lesions.

Based on cultural, morphological and biochemical characters study on 26 isolates of *C. pseudotuberculosis equi*, all isolates are Gram-positive, non-sporulated, non-motile pleomorphic, curved rods, catalase, urease and nitrate reduction positive which is specific reaction differentiate biotype 2 (buffalo origin) from biotype 1(sheep origin). These results exactly agrees with that obtained by **Zaki (1999)** who isolated *C. pseudotuberculosis* from samples collected from oedematous lesions in buffalo at El-Minia governorate by a ratio of 41.5% and **Selim (2001)** who has been studied OSD in buffalo and he recorded that, acute endemic disease in Egypt caused by C. *pseudotuberculosis equi* which characterized by the development of diffused swelling in the skin of the hind quarter, for limbs, belly and brisket regions these lesions usually affect the lymph nodes which become enlarged, inflamed and filled with pus due to contamination with other bacteria as *staphylococcus, streptococcus, E. coli* and *pseudomonas*.

Our study was nearly agree with that obtained by **Selim (1999)** and **Sohier *et al.* (2013)** who isolated 2 strains of biotype 2 from exudates of subcutaneous abscess of buffaloes aged 1-6 years by the ratio of 25% .We were completely disagree with state by **Arafa (2019**) who has been studied 43 buffalo cows hosted and owned sporadically during buffalo OSD spread in a village affiliating to Assiut governorate from 43 buffalo cows (had closed lesions either edematous or nodular) and a buffalo cow (had open ulcerative lesion), *C. pseudotuberculosis equi* (*C. ps. equi*) as 72% and *C. pseudotuberculosis ovis* (*C. ps. ovis*) as 28% were isolated and identified, and **Mohammad (2001)** and **Syame (2002)** who isolated *C. pseudotuberculosis* from skin lesion of buffaloes by ratio 14.3% and 14% respectively.

Synergistic Hemolytic Activity of *C. pseudotuberculosis* and *R. equi* culture filtrate (Modified CAMP) results:

All the *C. pseudotuberculosis* isolated from buffaloes tested in this study showed synergistic hemolysis with *R. equi* culture filtrate when

streaked onto Luria-Bertani (LB) agar containing 5% sheep erythrocytes and 10% *R. equi* culture filtrate. Clear zone of hemolysis surrounded the tested colonies as their diameter and intensity of which depend upon the extent of toxin production of the tested isolate.

Inhibition of Staphylococcal Hemolytic Activity with PLD (Reverse CAMP Test): The exotoxin producer strain of *C. pseudotuberculosis* inhibited the hemolytic activity of vertical streaked colonies hemolysis produced by staphylococci that appear as a wide clear zone of growth inhibitor between the two streaked strains.

Concerning their biological activities, all isolates were subjected to modified CAMP and reverse CAMP tests and all isolates showed large hemolytic zones around the growing colonies in the presence of *R. equi* and inhibit the hemolytic activity of *S. aureus* B lysine respectively, and these results were agree with **Songer *et al.* (1990)** and **Egen *et al.* (1989)**. All strains of *C. pseudotuberculosis equi*, produce a powerful exotoxin, this exotoxin is a phosphatidyle choline phosphatidohydrolase, better known as phospholipase D it functions as a sphingomyelinase, catalyzing the dissociation of sphingomyelin, an important membrane component, into ceramide phosphate and choline. *C. pseudotuberculosis* phospholipase D inhibits haemolysis produced by the beta-lysine of coagulase-positive *Staphylococcus* spp., which is also a sphingomyelinase, by competitively occupying the target site on the erythrocyte membrane. It properties has been utilized in the anti-betahemolysis inhibition (AHI or BHI) test for detection of antibodies to the exotoxin **(Muckle and Gyles, 1982).**

Synergistic hemolysis occurs when the *C. pseudotuberculosis* phospholipase D acts in concert with certain types of phospholipase C (exotoxin of *Rhodococcusequi*). This synergic hemolysis by the *C. pseudotuberculosis* toxins is useful in the identification of *corynebacterial* species and constitutes the basis of the synergic hemolysis inhibition (SHI) test. Our findings also supported the findings of **Radostits *et al.* (2007)** and **Mamman *et al.* (2011)** who reported that, the organism possesses a cytotoxic surface lipid coat that appears to facilitate intracellular survival and abscess formation. It also produces a phospholipase exotoxin that increases vascular permeability, has an inhibitory effect on phagocytes and may facilitate spread of infection in the host.

Application of antimicrobial sensitivity test for all *C. pseudotuberculosis equi* isolates: Table (3) revealed that, most isolates are sensitive toamoxicillin/ clavulanicacid (96.1%),and trimethoprim/ sulphmethazole (92.3%) followed by enerofloxacin, erythromycin (84.6% and 80.6%) respectively. On the other hand the tested isolates are highly resistant to ampicillin (92.3%), and nalidixic acid (88.5%).

Table (3). Antimicrobial susceptibility of *C. pseudotuberculosis equi* isolated from OSD lesions (26 isolates).

Antimicrobial drugs	Sensitive isolates		Resistant isolates	
	No	%	No	%
Amoxicillin/clavulanic acid	25	96.1	1	3.9
Ampicillin	2	7.7	24	92.3
Enerofloxacin	22	84.6	4	15.4
Erythromycin	21	80.6	5	19.4
Gentamycin	19	73.1	7	26.9
Kanamycin	21	80.6	5	19.4
Nalidixic acid	3	11.5	23	88.5
Penicillin	11	46.2	15	53.8
Streptomycin	8	30.8	18	69.2
Trimethoprim/Sulphamethazol	24	92.3	2	7.7

Varied sensitivity of *C. pseudotuberculosis* to different antimicrobials has been reported elsewhere **(Sargison, 2003**; **Connor *et al*., 2007**and **Abebe *et al.*, 2015**). **(Shawky *et al*., 2019**) revealed that, *C. pseudotuberculosis* were become more resist to some drugs when one or more other drug resistance genes are expressed on the same strain also Trimethoprim/ Sulphamethazole, aminoglycosides (gentamycin) and macrolide (erythromycin) are reported as the first-line antimicrobials to the effective therapy of generalized bovine skin infection based on modified in vitro disk diffusion method.

Table (4) showed that, the results of molecular detection of *C. pseudotuberculosis equi* virulence genes was evidenced in 6 samples of bacteriologically diagnosed isolates as *C. pseudotuberculosis equi* revealed that, all isolates positive to PCR-amplified DNA fragment of 203 bp and specific for the *Pld* gene of *C. Pseudotuberculosis equi* (Figure-1),PCR amplified DNA fragment of 406 bp, and specific for the *rpoB* gene (Figure-2) and PCR-amplified DNA fragment of 490 bp gene and specific for the *sigE* gene (Figure-3), while one isolate negative for the *sigE*gene. Also from our study all isolates were investigated for phospholipase–D activity by modified CAMP and reverse CAMP and showed positive results, this proved that PLD is a characteristic product of *C. Pseudotuberculosis equi* isolates.

Table(4). Detection of virulence genes in *C. pseudotuberculosis equi* isolates.

Number of tested strains	Virulence genes		
	sigE	***RpoB***	***Pld***
1	-	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+

+means presence of genes - = means absent of genes

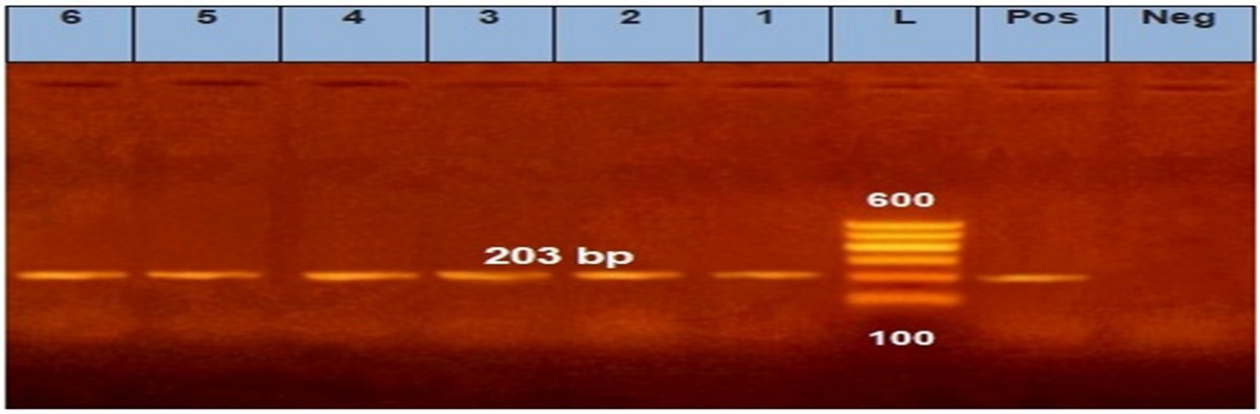

Fig. (1): Detection of virulence gene (*pld*) of *C. pseudotuberculosis equi* by polymerase chain reaction (PCR). **L:** DNA ladder (100 -600 bp.), **Pos:** Positive control. **Neg**: Negative control. **Lane's arrangement from (1 - 6)**: showing (*pld*) gene amplifications (203bp.) for six C. *pseudotuberculosis equi* isolates respec-

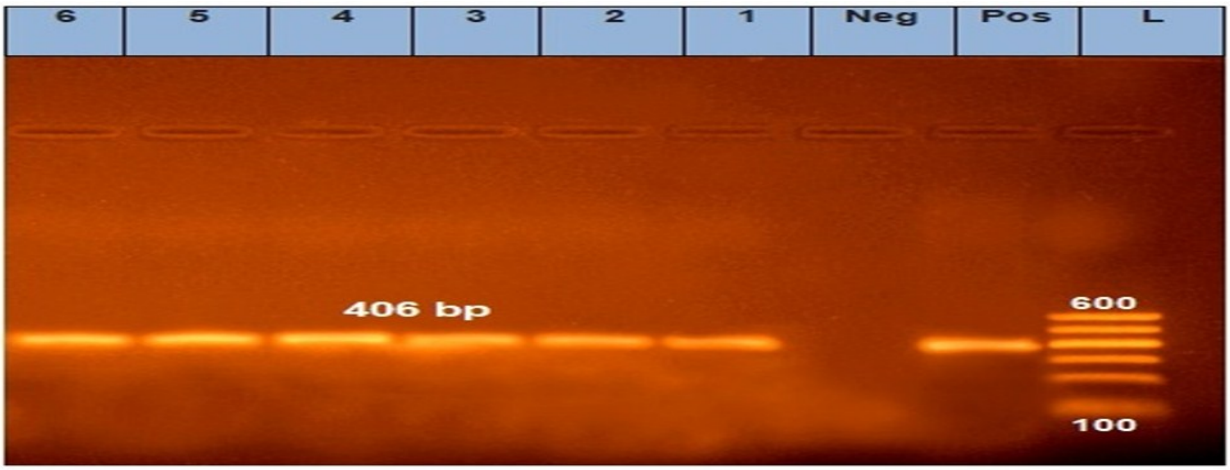

Fig. (2): Detection of virulence gene (*rpoB*) of *C.ps. equi* by polymerase chain reaction (PCR). **L**: DNA ladder (600 -100 bp.). **Pos:** Positive control**. Neg:** Negative control**. Lane's arrangement from (1 - 6):** showing (*rpoB*) gene amplifications (406bp) for six *C. pseudotuberculosis equi* isolates respectively.

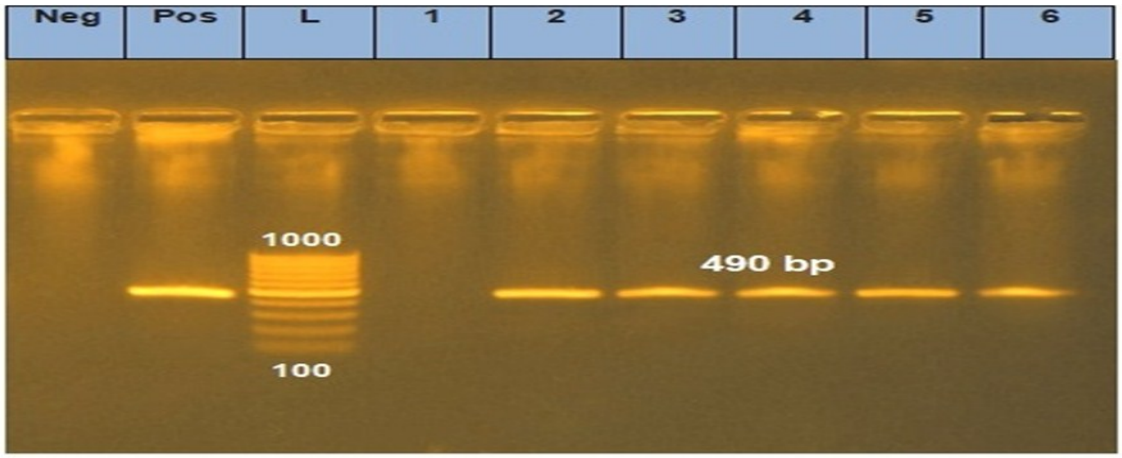

Fig. (3): Detection of virulence gene (*sigE*) of *C. pseudotuberculosis equi* by polymerase chain reaction (PCR). **L:** DNA ladder (1000 -100 bp.). **Pos**: Positive control. **Neg**: Negative control. **Lane's arrangement from (2-6):** showing (*sigE*) gene amplifications (490bp.) for six *C. pseudotuberculosis equi* isolates respectively and lane (1) showed no present of sigE.

These results agree with **Dorella (2006)** who reported that, virulence factors play an important role in the adhesion, invasion, colonization, spread inside the host and immune system evasion of pathogenic bacteria; they also allow contact, penetration and survival inside the host and (Phospholipase D) *PLD* gene increase vascular permeability and bacterial survival in the host.

The oligonucleotide primers used for PCR were from the *pld* gene of *C. pseudotuberculosis* (Phospholipase D), *PLD* gene protects the bacteria from killing by phagocytic cells and enables bacteria to escape from neutrophils and impair neutrophils chemo taxis toward the site of infection, so Phospholipase D (PLD) has for some years been implicated as the major virulence factor of *C. Pseudotuberculosis* **(Hodgson *et al.,* 1999)**. Also **Nabih *et al.* (2018)** was examined the isolated and identified *C. pseudotuberculosis* for evidence of virulence genes Phospholipase D (*pld*) and β-subunit of RNA polymerase (*rpoB*) by polymerase chain reaction (PCR). Pld gene-based PCR is more reliable than *rpoB* gene based ones for the diagnosis of *C. pseudotuberculosis Pld* gene based PCR is more reliable than *rpoB* gene based ones for the diagnosis of *C. pseudo tuberculosis.*

Bacteria can respond to different environmental stimulate through switching of the primary sigma (σ) factor subunit that is associated with core RNA polymerase by alternative σ factors. This confers novel promoter recognition specificities to the polymerase and provides same mechanism for rapid regulation of different sets of stress-responsive genes **(Staron *et al.*, 2009**). Proteins of the extra cytoplasmic function (ECF) family of alternative σ factors such as σE, mostly regulate response to cell surface stresses and have already been shown to control virulence associated genes indifferent pathogenic bacteria **(Kazmierczak *et al.,* 2005)**. Previous studies on *Mycobacterium tuberculosis* transcriptional responses to the stress conditions found within phagocytic cells have suggested the involvement of the ECF sigma factorσEin bacterial intracellular survival **(Fontán *et al.,* 2008)**. Moreover, sig E has recently been shown to be part of a small set of *M. tuberculosis* genes that are selectively unregulated in response to nitrosative stress **(Voskuil *et al.,* 2011)**. Also **Pacheco *et al.* (2012)** study with *C. Pseudotuberculosis*, he shows that, σE is indeed required for resistance to in vitro generated, biologically relevant concentrations of nitric oxide (NO). Moreover, he also demonstrate that this regulatory protein plays a role in resistance to combine NO/ peroxide stress faced by bacteria during infect.

Conclusion and Recommendation

*The results of this study suggested that *C. pseudotuberculosis equi* isolates obtained from buffaloes showing clinical symptoms of the oedematous skin disease may be occur contamination with other bacteria as *staphylococcus* and *streptococcus* species in single or mixed infection. *C. pseudotuberculosis equi* tend to carry more than one virulent gene so can produce several types of toxins having synergistic or cumulative gene in action leading to the acute form of the disease and their-severity of virulence depends upon the number of factors in the same strain which may explain the variety in clinical picture of the OSD, also our results showed that, amoxicillin/ clavulanic acid is the drug of choice for treatment of buffaloes infected with OSD.

**Strict hygienic measures should be periodical application of strong disinfectants to get rid pathogenic agents.

***Avoidance of miss-use of antibiotics either local or systemic because it resulted in increasing resistance of bacteria to the used antibiotics. Use of antibiotics should be according to the laboratory result.

****Periodical vaccination against viral diseases as lumpy skin disease.

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