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Molecular Characterization and Antibiotic Susceptibility of *Corynebacterium pseudotuberculosis* Isolated from Sheep and Goats Suffering from Caseous Lymphadenitis

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Abstract

Caseous lymphadenitis is a highly prevalent worldwide disease of sheep and goats caused by *C. pseudotuberculosis*, resulting in significant economic losses. In order to investigate the prevalence as well as the molecular characterization of *C. pseudotuberculosis* and monitoring the antibiotic susceptibility of the isolated strains, a total of 126 pus samples were collected aseptically from 80 sheep and 46 goats suffering from abscessation. The collected samples were subjected to bacteriological examination where the prevalence of *C. pseudotuberculosis* was 43.8% and 36.9% in diseased sheep and goats, respectively. The antibiotic sensitivity test was carried out using disc diffusion method, most of the isolates were resistant to penicillin (96.2%) and erythromycine (92.3%), highly sensitive to ciprofloxacin (96.2%), amikacin (90.4%) and neomycin (88.5%). Polymerase chain reaction was applied on 30 *C. pseudotuberculosis* isolates for the amplification and detection of both 16S rRNA and the RNA polymerase β-subunit gene (*rpo*B) genes to confirm the diagnosis of the isolates. The results revealed that all the tested isolates were positive for both genes. In conclusion, the incorporation of both phenotypic and genotypic characterization of *C.pseudotuberculosis* is more valuable and accurate for identification of isolates. Moreover, PCR is a fast and specific diagnostic tool used for the genetic analysis of *C. pseudotuberculosis.*

Keywords: Caseous lymphadenitis, *C. pseudotuberculosis*, 16S rRNA, *rpo*B

Introduction

Caseous lymphadenitis is a chronic debilitating disease affecting sheep and goats and is characterized by fibrous encapsulated suppurative abscesses in the peripheral lymph nodes and visceral organs that incorporate yellowish green granular pus [1,2]. The disease results in severe economic losses to sheep and goat producers worldwide, in particular due to the reduction of milk yield, wool and meat production, decreasing the fertility of the affected animals and condemnation of portions of affected carcasses at slaughter [3,4].

Genus *Corynebacterium* is one of the members of the *Actinomycetes* group. Other Genera are also included in this group such as *Nocardia* and *Mycobacterium*. Although these genera belonged to the same group, they greatly differ in many characters including cell wall composition, the percentage of peptidoglycan and mycolic acid and the guanine and cytosine ratio in their genome.

The *Actinomycetes* group includes several species which have both medical and veterinary importance, such as *Mycobacterium tuberculosis* and *M. bovis*; the causative agents of human and bovine tuberculosis; and *C. pseudotuberculosis* which is the main causative agent of caseous lymphadenitis [1].

C. pseudotuberculosis is Gram-positive, pleomorphic, non-capsulated, non-motile, non sporulating, facultative anaerobic and intracellular microorganism with worldwide distribution [5,6].

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The microorganism is classified into two biovars, the biovar *Ovis* (nitrate reduction negative), which mainly affects sheep and goats causing caseous lymphadenitis and the biovar *Equi* (nitrate reduction positive), which mainly affects horses and cattle causing ulcerative lymphangitis. The existence of these two biovars has been confirmed by biomolecular techniques [7,8].

Caseous lymphadenitis is mainly transmitted through abscess rupture which results in discharge of large numbers of *C. pseudotuberculosis* which contaminate the sheep fleece, and in turn the surrounding environment became contaminated. The transmission of infection to other animals could be either by direct contact with the diseased animal or indirect contact with the contaminated environment [5].

Caseous lymphadenitis diagnosis mainly depends upon the disease clinical characteristics and the isolation and identification of the causative agent from pus discharge. Molecular typing of *C. pseudotuberculosis* is necessary to confirm the diagnosis of caseous lymphadenitis. Polymerase chain reaction (PCR) is a reliable, accurate and rapid technique if compared with the conventional techniques used for isolation and identification of bacterial pathogens. PCR detection and amplification of 16S rRNA and the RNA polymerase β-subunit gene (*rpo*B) genes facilitates the differential diagnosis of *C. pseudotuberculosis* from other bacterial pathogens that may be present in pus samples [1].

This study was planned to investigate the prevalence of *C. pseudotuberculosis* affecting sheep and goats, monitoring the antimicrobial susceptibility of the isolated strains and PCR based detection of 16S rRNA and *rpo*B genes in order to confirm the diagnosis of the isolated *C. pseudotuberculosis* strains.

Material and Methods

Sampling

A total of 126 pus samples were collected aseptically from 80 sheep and 46 goats suffering from abscessation (clinically examined for the presence of abscess in the superficial lymph nodes including pre-scapular, pre-femoral, sub-mandibular and parotid lymph nodes) at different farms in Menoufia Governorate, Egypt. Pus samples were aseptically collected from opened abscesses by sterile swabs. The collected samples were kept on ice and brought to the lab as soon as possible.

Isolation and identification of C. pseudotuberculosis

Pus samples were directly inoculated onto blood agar base (Oxoid) supplemented with 5% defibrinated sheep blood. Plates were incubated at 37°C for 48-72 h, suspected pure colonies (yellowish white, opaque, hemolytic and convex colonies) were identified morphologically using Gram's stain as well as biochemically (catalase and urease activities, nitrate reduction and carbohydrates fermentation including glucose, lactose, maltose and galactose) using methods described by Quinn *et al.* [9].

Antimicrobial susceptibility testing by disc diffusion method

The susceptibility to eight antibiotics (penicillin, amikacin, neomycin, erythromycine, ciprofloxacin, sreptomycin, novobiocin and methicillin) was tested according to the National Committee for Clinical Laboratory Standards (NCCLS, 2013) using disc diffusion technique [10]. The susceptibility of the strains was determined according to the size of the inhibition zone.

*PCR detection of 16SrRNA gene and rpo*B *genes of C. pseudotuberculosis*

Randomly chosen 30 isolates were subjected to PCR for the detection of 16S rRNA and *rpo*B genes.

DNA extraction

Extraction of DNA from *C.pseudotuberculosis* strains by boiling method was carried out according to Faez *et al.* [11]. One ml of the bacterial broth culture was centrifuged at 5000 rpm for 5 min, the supernatant was discarded and the pellet was

re-suspended in 1 ml distilled water. Centrifugation at 5000 rpm for 5 min and washing in another 1 ml distilled water was repeated for three times. The pellet was re-suspended in 200 µl distilled water for boiling for 10 min. The suspension was then placed in ice for 5 min, and centrifuged at 10000 rpm for 5 min. The supernatant (containing the bacterial DNA) was then transferred to a fresh tube.

Amplification of the target genes

The amplification of 16S rRNA and *rpo*B genes was carried out according to Çetinkaya *et al.* [12] and Pacheco *et al.* [13], respectively, using specific primer sets Synthesized by metabion company, Germany were used (Table 1). The reaction was performed in a total volume of 50 µl containing PCR buffer [(50 mM Kcl, 10 mM tris - Hcl, 1mM $Mgcl_2$), dNTPS (Deoxy nucleotide Triphosphate) 200 uM, each (dATP, dGTP, dCTP and dTTP) (Advanced Bio enzymes ltd. UK), Two primer pairs each at 50 pmol/reaction, and 1.25 U of *Taq* DNA polymerase (Advanced Bio enzymes ltd. UK). Thermal cycling in a programmable thermal cycler (Coy corporation, Grass Lake, Michgan, USA) was done. A positive control was kindly provided by the Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia. Egypt. A negative control PCR reaction with no template was also included in this assay.

The reaction conditions for the amplification of 16S rRNA were initial denaturation (94ºC for 5 min), 30 cycles (denaturation at 94^{o}C for 1 min; annealing at 56 ^{o}C for 1 min; extension at 72^{o}C for 1 min) and final extension at 72°C for 5 minutes [12]. For the amplification of *rpo*B gene, the reaction conditions were initial denaturation (95ºC for 3 min), 35 cycles (denaturation at 95ºC for 1 min, annealing at 55ºC for 40s; extension at 68ºC for 1 min) and final extension at 68ºC for 5 min [13].

Table 1: Oligonucleotide primers used for PCR assay for *C. pseudotuberculosis*

Primer	Primer Sequence 5'-3'	Annealing temperature	PCR product (bp)	Reference
16S rRNA- F	ACCGCACTTTAGTGTGTGTG	56°c	815	[12]
16S rRNA- R	TCTCTACGCCGATCTTGTAT			
***rpo*B-F**	CGTATGAACATCGGCCAGGT	68°c	446	[13]
***rpo*B-R**	TCCATTTCGCCGAAGCGCTG			

Screening of the PCR products

Ten µl of the amplified PCR product was analyzed by electrophoresis in 2% agarose gel stained with 0.5 µg of ethidium bromide/ml (Sigma). Electrophoresis was carried out in 1X TAE buffer at 80 volt for 1 hour. Gels were visualized under UV transilluminator (UVP, UK) and photographed.

Results

Prevalence of C. pseudotuberculosis in diseased sheep and goats

C. pseudotuberculosis was isolated from 35 (43.8%) and 17 (36.9%) diseased sheep and goats, respectively, with an overall isolation rate of 41. 3% (Table 2). Seventy four samples (45 from sheep and 29 from goats) were negative for *C. pseudotuberculosis*, while, they were positive for other bacterial pathogens.

Antibiogram analysis of the isolated C. pseudotuberculosis strains

As shown in Table (3)**,** the antibiotic sensitivity test revealed that, most of the examined *C. pseudotuberculosis* isolates were highly resistant to penicillin (96.2%) and erythromycine (92.3%), while they were highly sensitive to ciprofloxacin (96.2%), amikacin (90.4%), neomycin (88,5%) and streptomycin (80.8%). Moderate sensitivity to novobiocin (86.6%) and methicillin (76.9%) was also observed.

Table 2: Prevalence of *C. pseudotuberculosis* isolated from diseased sheep and goats

Animal spp.	No. of pus samples	No. of *C. pseudotuberculosis*	% of *C. pseudotuberculosis*	No of samples positive for other bacterial pathogens
Sheep	80	35	43.75	45
Goats	46	17	36.95	29
Total	126	52	41.26	74

Table 3: Antibiogram of the isolated *C. pseudotuberculosis*

Antimicrobial agents	Resistant		Intermediate		Sensitive	
	No	%	No	%	No	%
Penicillin	50	96.2	2	3.9	-	-
Ciprofloxacin	-	-	2	3.9	50	96.2
Neomycin	-	-	6	11. 6	46	88.5
Amikacin	-	-	5	9.6	47	90.4
Novobiocin	7	13.5	45	86.5	-	-
Erythromycine	48	92.3	4	7.7	-	-
Sreptomycin	-	-	10	19.2	42	80.8
Methicillin	10	19.3	40	76.9	2	3.8

Molecular characterization of C. pseudotuberculosis

In the present work, thirty *C. pseudotuberculosis* isolates were subjected to PCR amplification of 16S rRNA and *rpo*B genes in order to confirm the bacteriological identification of the isolates. All the selected *C. pseudotuberculosis* isolates were positive for both 16S rRNA and *rpo*B genes (Figures 1 and 2).

Discussion

Caseous lymphadenitis is a chronic disease of veterinary importance affecting sheep and goats resulting in excessive economic losses of animal industry worldwide [3,4]. In the present study, the prevalence of *C. pseudotuberculosis* in the affected sheep and goats was 43.8% and 36.9%, respectively (Table 2).

C. pseudotuberculosis was reported from cases of abscessation in sheep and goats with the isolation rates of 26.3% and 45.5%, respectively, in Egypt [14]. Higher prevalence in sheep and goats (70%) was recorded in Patagonia [15]. In addition, in Turkey, 96 suspicious *Corynebacterium* isolates from sheep and goat abscesses were confirmed by 16S rRNA as *C. pseudotuberculosis* [12].

The usage of the barbed wire fences for guarding the sheep and goats farms is one of the maximum vital bad management practices that cause wounds with subsequent infection [16]. The disease is mainly transmitted through abscesses rupture and other animals could be subjected to *C. pseudotuberculosis* infection, either by direct contact with the diseased animal or indirect infection through the contaminated environment [5]. Caseous lymphadenitis has been reported in countries formerly free from the disease, which mainly attributed to importation of diseased cases [17]. *C. pseudotuberculosis* isolates showed resistance to penicillin (96.2%) and erythromycine (92.3%) and they were highly sensitive to ciprofloxacin (96.2%), amikacin (90.4%) neomycin (88.5%) and streptomycin (80.8%). Moderate sensitivity to novobiocin (86.6%) and methicillin (76.9%) was also observed (Table 3).

These results were in accordance with those obtained by Hatem *et al.* [14] in Egypt and Hassan *et al.* [18] in Saudi Arabia. In contrary, Abdel Wahab and Shigidi [19] reported that *C. pseudotuberculosis* isolates were highly sensitive to erythromycin and ampicillin. Based upon the results of several studies on different *Corynebacterium* spp. showing resistance to penicillins, it could be concluded that both resistance mechanisms occur in *Corynebacterium* spp., including synthesis of penicillinase enzymes and alteration of

penicillin-binding proteins [20]. Resistance to macrolides, (erythromycin), mainly occurred due to three major mechanisms: change of the ribosome binding site (23S rRNA) (methylation) or modification in the mode of active efflux of the antibiotic from the cell or antibiotic inhibition by enzymes [21,22].

Methylation prevents binding of the antibiotic molecules in the peptidyltransferase center within the large ribosome subunit. *Corynebacterium* spp. carry genes of class *erm* (erythromycin ribosome methylation), encoding the methylase enzyme, which causes methylation of 23S rRNA.

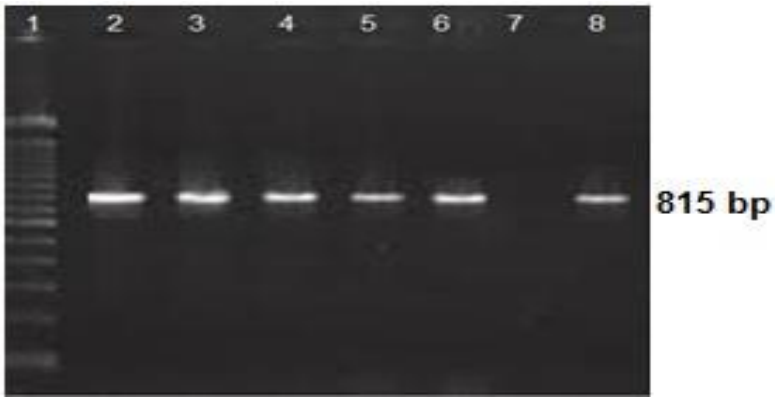

Figure 1: Electrophoretic pattern of 16S rRNA gene in 2% agarose gel showing the amplified product at 815 bp [lane (1): 100 bp DNA ladder, lanes (2-6): positive samples, lane (7): control negative, lane (8): control positive *C. pseudotuberculosis* strain].

The traditional bacteriological methods are not usually completely accurate, as a result, the development of a fast and specific diagnostic tool is vital for early diagnosis and control of caseous lymphadenitis [12].

In the current study, PCR was used for the detection and amplification of 16S rRNA gene in order to confirm the diagnosis of the isolated strains. The 16S rRNA gene, the gene of choice for most microbial taxonomy studies, therefore, this gene is useful for estimating the prevalence of *C. pseudotuberculosis* in the animals studied [12]. All the selected *C. pseudotuberculosis* isolates were positive for 16S rRNA gene with the specific 815 bp amplicon size (Figure 1). In addition, amplification of *rpoB* gene in the obtained isolates was also carried out (Figure 2).

All the examined isolates were positive with the specific amplicon size (446 bp). these results agreed with those reported by other studies [13,23]. PCR detection of both 16S rDNA and *rpo*B genes help in the differential diagnosis of *C. pseudotuberculosis* from other pyogenic pathogens that might present in pus discharge [13].

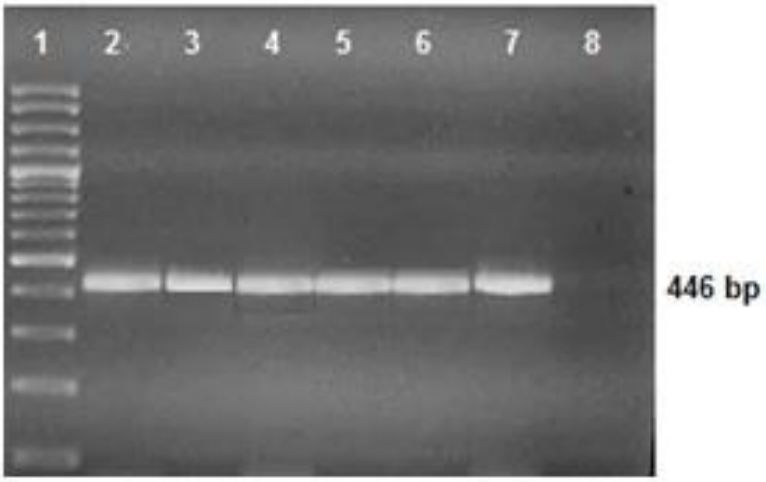

Figure 2: Electrophoretic pattern of *rpo*B gene in 2% agarose gel showing the amplified product at 446 bp [lane 1: 100 bp DNA ladder, lane (2): control positive *C. pseudotuberculosis* strain, lanes (3-7): positive samples, lane (8): control negative].

Similarity in the sequence of 16S rRNA (99.7%) and *rpoB* gene (93.6%) between *C. pseudotuberculosis* and *C. ulcerans* have been previously reported [12,13]. However, *C. ulcerans* have not been isolated in small ruminants [12]. A study by Khamis *et al.* [24] on the sequence analysis of *rpo*B gene from different pyogenic bacteria reported that PCR of *rpo*B gene is a powerful diagnostic tool that can be used to accomplish the 16S rRNA gene characterization of *Corynebacterium* species from other pyogenic organisms [24].

Conclusion

In conclusion, incorporation of both phenotypic and molecular characterization of *C. pseudotuberculosis* is more reliable and more accurate for identification of the isolates. Continuous monitoring of the antimicrobial susceptibility is necessary due to the increased microbial resistance and for selection of the antibiotic of choice. PCR is a fast and specific diagnostic tool used for the characterization of *C. pseudotuberculosis.*

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

التوصيف الجزيئي و الحساسية للمضادات الحيوية لعترات وتدية السل الكاذب المعزولة من الاغنام والماعز التي تعاني من إلتهاب العقد الليمفاوية التجبني

عبدالعظيم محمد الجمال

قسم البكتريا والمناعة والفطـريات- كلية الطب البيطـرى- جامعة قناة السويس

إلتهاب العقد الليمفاوية التجبني (السل الكاذب) هو مرض واسع الانتشار في جميع أنحاء العالم ويصيب الأغنام والماعزويسببه ميكروب وتدية السل الكاذب، مما يؤدي إلى خسائر اقتصادية فادحة. لتحديد مدى انتشار وتدية السل الكاذب بالاضافة الى التوصيف الجزيئي للميكروب ورصد حساسية العترات المعزولة للمضادات الحيوية، تم تجميع ١٢٦ عينة من القيح تحت ظروف معقمة من ٨٠ من الأغنام و ٤٦ من الماعز والتي تعاني من خراريج صديدية .ثم خضعت العينات التي تم جمعها للفحص البكتريولوجي حيث كانت نسبة عزل ميكروب السل الكاذب في الاغنام المصابة ٤٣,٨٪ وفي الماعز ٣٦,٩٪. و قد تم أجراء اختبار الحساسية للمضادات الحيوية باستخدام طريقة الانتشار من الأقراص حيث كانت معظم العترات المعزولة مقاومة للبنسيلين (٩٦,٢٪) والاريثروميسين (٩٢,٣٪) وحساسة للغاية للسيبروفلوكساسين (٩٦,٢٪) والأميكاسين (٩٠,٤%) والنيوميسين (٨٨,٥٪) .وقد تم استخدام تفاعل البلمرة المتسلسل للكشف عن تواجد جينات (16S rRNA) و(*rpo*B) في ٣٠ من العترات المعزولة لتاكيد تشخيصها حيث كانت جميعها موجبة لكلا الجينين. من نتائج الدراسة يستنتج أن استخدام التحليل المظهري والجيني معا لميكروب وتدية السل الكاذب هو أداة قيمة لتوصيف العترات المعزولة. كما يعتبر تفاعل البلمرة المتسلسل أداة تشخيصية قيمة وسريعة ومحددة تستخدم للتوصيف الجيني للميكروب.