Research Paper

Arcanobacterium pyogenes infected gazelles at King Khalid Wildlife Research Centre, Thumamah, Kingdom of Saudi Arabia

ABSTRACT

The genus *Archanobacterium* was studied according to its cell wall constituents and the building of the respiratory quinines to house bacterial strains associated to the species of *Corynebacterium haemolyticum.* This research was carried out to identify phenotypic and genotypic properties of *Arcanobacterium pyogenes* found in infected lungs after death from gazelles kept at Thumamah, Riyadh Saudi Arabia. Out of hundred *Gazella gazella* 35% were positive and *Gazella marica* (45%), while 20% of *G. gazelle* and *G. marica* were negative. Bacteria from lung lesions of *G. gazella* and *G. marica* were isolated using 5% sheep blood agar. It was sub-cultured several times on 5% blood agar plates until single colonies were obtained. Bacterial DNAs were extracted from the single colonies obtained by using Qiagen DNeasy Blood and Tissue Kit (QIAGEN GmbH, Hilden) following the manufacturer's instructions. The primers used were very specific and gave rise to magnification of approximately 1400 bp. Purified DNA was amplified by using 16S rDNA and beta subunit of RNA polymerase coding gene *rpo*B of the 10 isolates from species were amplified by the oligonucleotide primers described Khamis and a species precise primers of the 16S-23S rDNA intergenic piece region (ISR). All PCRs were performed using a MBS ThermoHypaid PCR machine. Neighbour joining and maximum likelihood (ML and NJ consensus) tree based on 419 bp fragments of 16S rRNA members' genes of *A. pyogenes* species were constructed.

Key words: *Arcanobacterium*, gazelles, lung, lesions, pyogenes.

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INTRODUCTION

Elling et al. (2016) reported that ubiquity of saturated menaquinones of the *Archaea* compared to bacteria suggested that these compounds may represent an ancestral and diagnostic feature of the *Archaea. Arcanobacterium* means 'mysterious bacterium', which an epithet is quite befitting for an organism that is frequently ignored by the Clinical Microbiologists, because of its deemed status as pollutant or regular flora (Vanessa et al., 2011). Several patients had concomitant Epstein-Barr virus (EBV) or a bacterial coinfection and was theorized that a patient's compromised immune system allows the organism to become pathogenic (Bridgette et al., 2017). Cultures on blood agar yielded heavy growth of *Arcanobacterium pyogenes* and *Enterococcus faecalis*, both identified by standard biochemical methods (Kannaiyan et al., 2010). Post-partum uterine infection causes infertility and economic losses in the dairy industry (Ajevar et al., 2013).

The proof of identity of *C. urealyticum* is single of the main contests facing the laboratory. Phenotypic studies using home-made media or commercial systems (API Coryne) may remain for proper identification.

Identification can also be confirmed by polymerase chain reaction (PCR) (Reem et al., 2014). *A. pyogene* is a pathogen which looks for chance and in dairy, such as, beef cattle and gazelles is very essential. It is a mutual resident of the lubricated tissues of these animals (Mohammed et al., 2011). Molecular tool using 16S rdeoxyribonucleic acid (DNA) universal eubacterial forward primer 16F27 (5´-AGA GTT TGA TCC TGG CTC AG-3´) and reverse primer 16SrDNA R1525 (5´-AAG GAG GTG ATC CAG CCG CA-3´), MWG Biotech AG (Ebersberg, Germany) identified the isolates successfully as *A. pyogenes* (Omar, 2012). Unusual caudal vena cava thrombosis in a cow, secondary to Trueperella (*A. pyogenes*) infection was reported by Garcia et al. (2016).

 A. pyogenes residents in the mucous membranes of local animals, such as cattle, sheep, swine and goats cause bacteria infection to persons who live in rural areas because of underlying illnesses such as cancer and diabetes. A current writing assessment caused by a case of *A. pyogenes* endocarditis found 13 clear cases of human infection with this agent; many patients had a past of close interaction with domestic animals (Carlos et al., 2009).

 Forest musk deer (*Moschus berezovskii*) are lonely ruminants found mostly in South-West China. The musk secreted by the deer is a traditional valuable Chinese medicine; it is also used in the production of perfumes. The chemotaxonomic properties of strain Murakami[T] were determined using methods accepted for use with actinomycetes (Azume et al., 2009). Lately, *Trueperella* was suggested to include 5 species formerly categorized as belonging to *Arcanobacterium.* Among these reclassified species is *T. pyogenes*, earlier known as *A. pyogenes*, *Actinomyces pyogenes* and *Corynebacterium pyogenes*. *T. pyogenes* has long been known as a mucosal membrane resident in many animal species and as a pathogen that looks for any means to cause infection (Robert et al., 2014). *Arcanobacterial* infections can be seen in diverse animal species (cattle, pig and turkeys) but more rarely in rabbits. However, in the first description it was microbiologically identified as *A. pyogenes* and the causal agent was multinodular abscessation due to this germ in rabbits (Shahbazfa et al., 2013).

 Mixed bacterial infections in the feet of deer were also reported in free-living and captive animals (Santiago et al., 2004). Phylogenetic investigation of 16S rRNA gene sequences from strain Murakami[T] and other associates of the genus *Arcanobacterium* supported the phenotypic findings that strain Murakami[T] signifies a new species, for which the name *Arcanobacterium abortisuis* sp. nov. is proposed (Azume et al., 2009). Culture fluid from two year old pull infected by *Arcanobacterium* spp was identified as *Arcanobacterium haemolyticum* by using an API Coryne biochemical test strip (Bancroft-Hunt et al., 2010).

 A. pyogenes is a commensal and opportunistic pathogen of economically significant livestock, causing diseases as diverse as mastitis, liver abscessation and pneumonia. This bacterium has a number of virulence features that contribute to its pathogenic potentiality (Helen et al., 2005).

MATERIALS AND METHODS

Bacteria were isolated from lung lesions from *G. gazella* and *G. marica* by using 5% sheep blood agar incubated at 37°C. Five isolates were isolated from both animal species (3 from the former and 2 from the later). From isolated organisms single colonies were sub-cultured severally in 5% blood agar plates until pure homogeneous single colonies were obtained. The organisms were identified based on cultural characteristics, gram stain and biochemically using Api-Coryne test system (bioMereiu, Germany).

DNA extraction

Bacterial DNA from pure colonies was extracted using Qiagen DNeasy Blood and Tissue Kit (QIAGEN GmbH, Hilden) following the manufacturer's instructions. A volume of 180 µl of the lysis buffer was prepared using 20 mM Tris-HCl ph=8, 2 mM Sodium EDTA, 1.2% Triton X and 20 mg/ml of lysozyme. After the bacterial lysis all the rest steps were carried out following the producer's procedure.

PCR amplification

Universal primers were used to amplify the 16S rDNA (Reem et al., 2014). Additionally, the beta subunit of RNA polymerase encoding gene *rpo*B of the 10 isolates from both species were amplified using the oligonucleotide primers as described by Khamis et al. (2004) and species specific primers of the 16S-23S rDNA intergenic spacer region (ISR). All PCRs were performed using a MBS ThermoHypaid PCR machine. The primers used in this study were manufactured by MWG Biotech (Germany). All reactions were done in a 25 µl reaction volume with 0.5 units of *Taq* polymerase (Bioline, UK), 2 m/mol MgCl$_2$, 40 µm of each dNTP and 200 n/mol of each primer. Amplifications were performed with a cycle profile of 92°C for 30 s, 35 cycles of 92°C for 30 s and annealing temperature was dependant on the primer used (Table 1) for 30 s and 72°C for 30 s and final extension cycle of 72°C for 5 min. All PCR reactions were loaded into 1.5% agarose gel (Agarose NA GE Healthcare Bio-Sciences AB SE-751 84 Uppsala) stained with Ethidium Bromide (Sigma Chemical Co.) and seen under ultra-violet lights using gel documentation system (Syngene, UK). DNA bands were excised using a sterile scalpel blade. Qiaquick Gel Extraction Kit (QIAGEN GmbH, Hilden) were used to collect the DNA from the excised gel using the producer's

Table 1: Species specific primers used, the oligonucleotide primer sequences, the annealing temperatures as well as the expected amplicon size in base pairs. All PCRs were performed using a MBS ThermoHypaid PCR machine. The oligonucleotide primers used in this study were synthesized by MWG Biotech (Germany).

Primer name	Primer sequence 5'-3'	Annealing temperature	PCR Product (bp)
16S rDNA UNI-L	AGA GTT TGA TCA TGG CTC AG'	58	1400
16S Rdna UNI-R	GTG TGA CGG GCG GTG TGT AC		
16S-23S-ISR (A. pyogenes)	GTT TTG CTT GTG ATC GTG GTG GTT ATG A	64	122
16S-23S-ISR (A. pyogenes)	AAG CAG GCC CAC GCG CAG G		
plo-Pyolysin 5A	CGA TCC CTC TGG TGT ACT TGC	60	704
plo-Pyolysin 5B	GCT TGA CAA AAA TCT GGC GTC C		
plo-Pyolysin 6A	GGC CCG AAT GTC ACC GC	55	270
plo-Pyolysin 6B	AAC TCC GCC TCT AGC GC		
rpoB-C2700F	CGW ATG AAC ATY GGB CAG GT	37 and 50	446
rpoB-C313R	TCC ATY TCR CCR AAR CGC TG		

directions. Sequencing of PCR fragments were conducted using the same PCR primers for PCR amplification using the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit (GE Healthcare UK Limited Little Chalfront Buckinghamshire HP7 9NA UK). Sequences were run on ALFexpress II (GE Healthcare).

RESULTS AND DISCUSSION

Bacteria isolated from infected lung tissues of both *Gazella arabica* and *G. marica* showed colonies surrounded by a narrow sharp hemolytic zone, gram stain of the organisms revealed gram positive short rods which were suggestive of *A. pyogenes*. Additional confirmation was attained after biochemical tests. Table 1 shows the oligonucleotide primer and the annealing temperatures as well as, the expected amplicon size in base pairs. Purified DNA was amplified using primers which amplify the 16S rDNA using the universal primers (Reem et al., 2014). Beta subunit of RNA polymerase encoding gene *rpo*B of the 10 isolates from both species were amplified using the oligonucleotide primers as described by Khamis et al. (2004) and species specific primers of the 16S-23S rDNA intergenic spacer region (ISR). All PCRs were performed using a MBS ThermoHypaid PCR machine. The PCR products result from amplification of the DNA yielded the expected size for the 16S and a fragment approximately 1400 bp was obtained and a partial sequence of 455 bp generated. With regard to 23 S, Pyolysin 5, Pyolysin 6 and RPOB sequences of 91, 417, 250 and 396 bp were respectively obtained. Resultant sequences were aligned with the relevant bacterial sequences available in the Genbank and showed resemblance to *A. pyogenes* (*Truperella pyogenes*) sequences. Figure 1 shows ML and NJ consensus tree based on 419 bp fragments of 16S rRNA gene of members of *Trueperella* closest species. Maximum likelihood (ML) consensus tree from concatenated (16S + RPOB50C + RPOB37C + Pyolsin 5 + Pyolsin 6) sequences of *Trueperella* isolates were obtained. Figure 2 shows bootstrap percentages from ML analysis. Figure 3 shows the 16S rDNA UNI, RpoB 37C, RPBO 50C, 16S-23S, PLO5 and PLO6 genes of *A. pyogenes.*

A. pyogenes was lately reclassified from the genus *Actinomyces* the major animal pathogen within the *Arcanobaium* genus. Although human infections caused by this organism have been reported but the validity of these reports is questionable due to lack of microbiological data ensuring a definite distinction of this agent from closely related bacteria such as *Arcanobacterium haemolyticum*. Historically, *A. pyogenes* was dismissed as a commensal or a co-invader, rather than an organism with substantial pathogenic potential. This is similar to the way that *Streptococcus aureus* was discarded as a mere skin contaminant in human diagnostic laboratories several decades ago. In fact *A. pyogenes* possesses a large virulence determinant, including potent PLO pyolysin genes, numerous mechanisms for host cell adhesion and the ability to invade and survive within epithelial cells and professional phagocytes, along with other putative virulence factors, such as proteases. Furthermore, *A. pyogenes* causes a variety of infections in diverse animal hosts which are seldom seen in other pathogenic bacteria. Such diversity highlights the possibility that *A. pyogenes* strains may be differentially equipped with virulence attributes, or possibly, their genetic regulation, leading to differential expression *in vivo* (Helen et al., 2005).

In this research, *A. pyogenes* was isolated and identified from lung lesions of *G. gazella* and *G. marica*. Number of assays such as cell culture method, ELISA, RPLA and
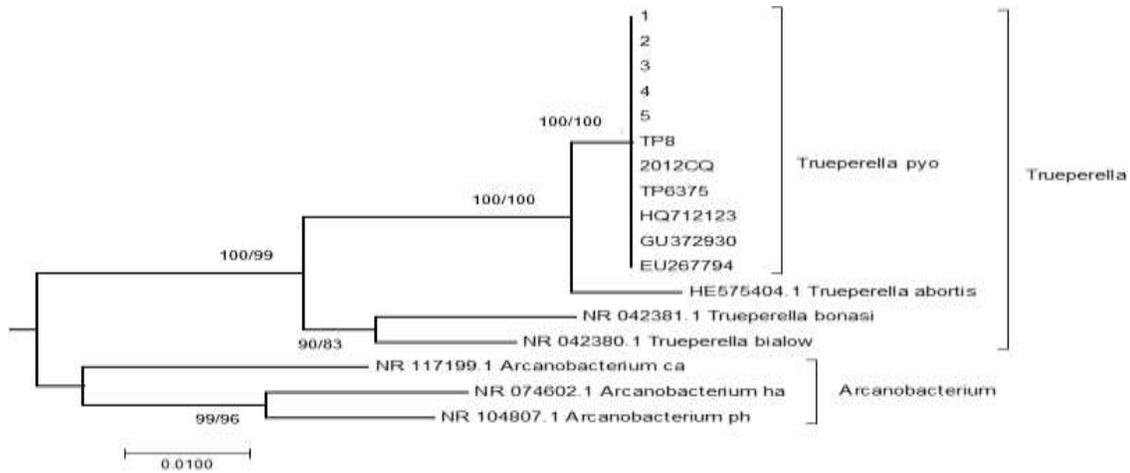
Figure 1: ML and NJ consensus tree based on 419 bp fragments of 16S rRNA gene of members of *Arcanobacterium pyogenes* species. Numbers given at nodes of branches are the bootstrap values for ML and NJ analysis, respectively. Out group: *Actinomyces bovis* 16S rRNA gene 1,434 bp linear DNA (X81061), *Actinobaculum schaalii* 16S rRNA gene 1,485 bp linear DNA (Y10773), *Actinomyces radicidentis* strain CCUG 36733 and complete genome 3,051,613 bp (CP014228).
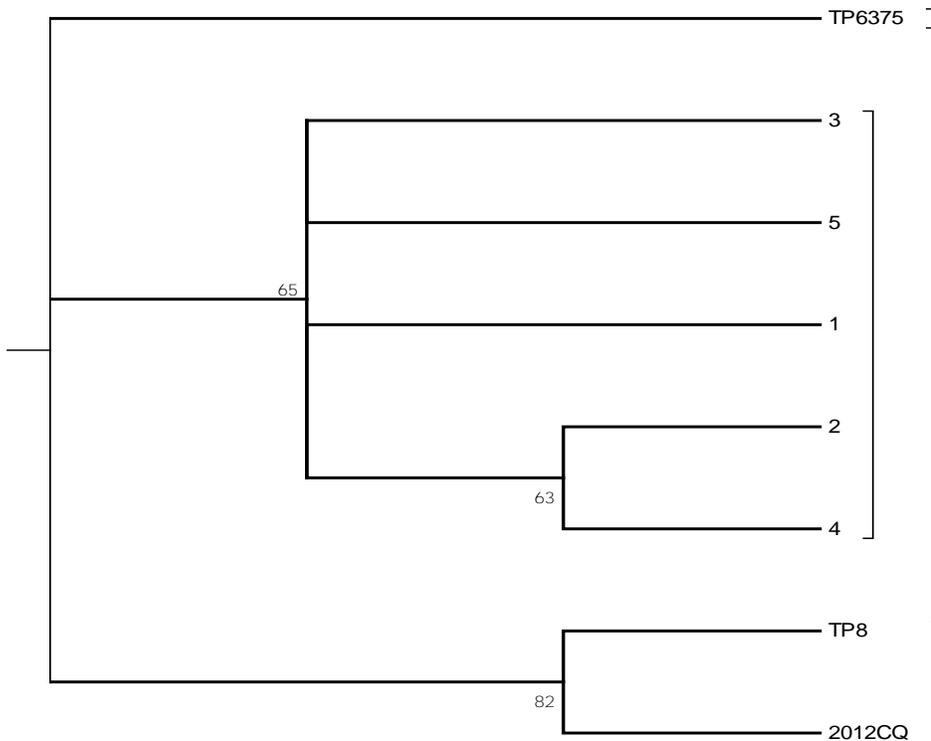

Figure 2: Maximum likelihood (ML) consensus tree from concatenated (16S + RPOB50C + RPOB37C + Pyolsin 5 + Pyolsin 6) sequences of *Trueperella* isolates. Bootstrap percentages from ML analysis are shown at nodes.

hybridization method were made available for the identification of *A. pyogenes* infection. The cell culture method was sensitive but this approach is laborious, time-consuming and requires several days to identify the pathogen. The ELISA method is sensitive and a rapid diagnostic tool but it requires monoclonal antibodies which are expensive.

Hybridization procedure is a very sensitive and effective molecular technique for specific detection of the biological agents according to their genetic structure. It is effectively
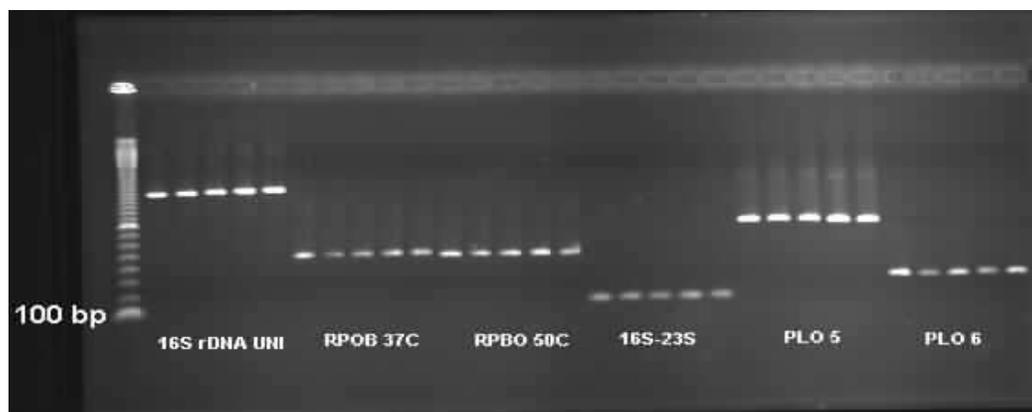
Figure 3: 16S rDNA UNI, RpoB 37C, RPBO 50C, 16S-23S, PLO5 and PLO genes of *Arcanobacterium pyogenes*.

used for the identification of pathogen; however, it has got some limitations, including the risk of using radioactive probes, complexity, time-consuming and the lack of its efficiency for some clinical samples in medical diagnostic laboratories. Genes of *A. pyogenes* was successfully isolated and ML and NJ consensus tree made based on 419 bp fragments of 16S rRNA gene of members of *A. pyogenes* species.

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