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Research Article

GENOTYPING AND GENETIC DIVERSITY OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* STRAINS ISOLATED FROM CASEOUS LYMPHADENITIS IN SHEEP AND GOAT

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ABSTRACT: A high level of genetic diversity was present in *C. pseudotuberculosis* strains in Duhok, Iraq. ERIC-PCR is a valuable technique for epidemiological studies. Strains of *C. pseudotuberculosis* from sheep were genetically diverse from that of goat. The ERIC PCR was used to fingerprint 22 strains of *C. pseudotuberculosis* obtained from mediastinal lymph nodes of probable caseous lymphadenitis infections in sheep and goat. Previously, conventional microbiological and molecular techniques were used to identify these isolates. Compared to *rpoB* gene sequencing, ERIC-PCR typing revealed that 22 strains of *C. pseudotuberculosis* were categorized into 13 ERIC types (genotypes). Genotypes 8, 9 and 12 signified the most predominant clones. The majority of sheep strains were more diverse than goat strains. The current study's findings indicate that different *C. pseudotuberculosis* clones circulate in the Duhok abattoir, and genotyping *C. pseudotuberculosis* strains using ERIC-PCR is essential for determining the evolutionary genetics of the species and for molecular epidemiology studies when compared to certain other molecular typing techniques.

Key words: *Corynebacterium pseudotuberculosis,* ERIC-PCR, Epidemiologic relationships, Genotyping.

INTRODUCTION

Caseous lymphadenitis (CLA) in goats and sheep is caused by *Corynebacterium pseudotuberculosis* (Guimarães *et al*. 2011a), in which abscessation in superficial and visceral lymph nodes is the usual characteristics resulting in severe decreases in animal performance (Baird and Fontaine 2007). The rate of *C. pseudotuberculosis* infection in sheep and goat in Duhok, Iraq, has greatly grown in recent years (Issa *et al*. 2021, Abdulrahman *et al.* 2020, Abdulrahman 2021). Numerous genotyping techniques have been proposed for the purpose of assessing the bacterial genetic diversity and epidemiological relation including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Abreu *et al*. 2008), ribotyping (Abdulrahman 2021), Pulse-Field Gel Electrophoresis (PFGE)(Connor *et al*. 2000) and Random Amplified Polymorphic DNA (RAPD) (Foley *et al*. 2004). All of the above stated typing techniques demonstrated a high degree of genetic similarity among the bacterial strains (Dorneles *et al*. 2012). A typing approach that relies on enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) was recently proposed, and it was proven to be an excellent indicator for the genetic discriminating of bacterial strains, with high resolution and repeatability (Guimarães *et al.* 2011b, Taha 2021). For such reasons, the current study used ERIC-PCR to study the genetic diversity and epidemiological connections among *C. pseudotuberculosis* isolates from sheep and goat with CLA slaughtered in Duhok abattoir, as well as to determine the efficacy of using ERIC-PCR rather than the *rpoB* gene sequencing to genotype those *C. pseudotuberculosis* strains.

MATERIAL AND METHODS

Bacterial strains and genomic DNA extraction

A total of 22 *C. pseudotuberculosis* isolates were used in this study to investigate their clonal relatedness and

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their genetic diversity using ERIC-PCR fingerprinting. These isolates were previously recovered from cheesy pus of enlarged lymph nodes from carcasses of suspected cases of CLA in sheep and goat in a Duhok abattoir. The isolates were recognized as *C. pseudotuberculosis* by phenotypic methods including typical colonies on blood agar (2-3 mm, cream colored colonies, with a narrow beta-hemolysis zone after 48h incubation), Gram-positive pleomorphic rods arranged as Chinese letters observed by Gram stained smear, catalase positive, ability to hydrolyze urea and tested for reverse CAMP test (inhibition of *Staphylococcus aureus* beta-hemolysis). The isolates were further confirmed by molecular method through PCR amplification of specific target genes including *16S rRNA*, RNA polymerase b-subunit (*rpoB*) and phospholipase D (*pld*) genes (Abdulrahman 2020). Unfortunately, we did not get any information about the geographical foundation of the isolates. It really should be mentioned, however, the slaughter house receives a variety of animal species for slaughtering from both the local area (Duhok province) and neighboring counties including Iran, Syria and Turkey. All of the 22 strains from both sheep and goat was probably classified as genetically related (grouped with in the same lineage) as indicated by the sequence analysis of the *rpoB* gene in a previous study by Abdulrahman (2021).

Genomic DNA was extracted usingthe thermal extraction method (Taha and Yassin 2019, Singh *et al*. 2019), with minor modification. Briefly, 100 µl of stock culture was inoculated onto blood agar containing 7% sheep blood. In 200 µl of sterile double distilled water, 2 to 3 pure colonies were homogenized. The suspension was vortexed for at least 15 seconds before being immediately heated at 95°C for 10 minutes; the materials were then chilled with ice before being centrifuged. One hundred fifty µl supernatant was used as a template DNA for PCR. The purity and concentration of extracted DNA were examined using a nanodrop (Thermo Scientific, USA)(Biswas *et al*. 2018). In the present study, among the 22 *C. pseudotuberculosis* samples, sample number 1 and sample number 4 were from goats. All others were from sheep.

ERIC-PCR fingerprinting

All isolates of *C. pseudotuberculosis* were subjected to ERIC-PCR to identify similar strains and distinguish different strains using the primer sequences (ERIC1: 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2: 5'-AAGTAAGTGACTGGGGTGAGCG-3') described by Versalovic *et al*. (1991). The PCR assays were carried out in a total volume of 25 µl. Each reaction consisted of 1 µl primers for each (10 pmol), 12 µl of hot start premix (Genedirex, Taiwan), 2 µl of sample DNA (30-100 ng/µl) and nuclease-free water (9 µl) (Qiagen, Germany) up to 25 µl (Taha 2021). The PCR reaction was conducted using the 9700 GeneAmp PCR system (Applied Biosystem, USA) according to the PCR program used by Bakhshi *et al.* (2018): the first denaturation was for 5 min at 94°C, next with 35 cycles of repeated steps each of 94°C for 1 min, 54°C for 1 min, and 72°C for 5 min. Finally, post PCR extension was done at 72^0C for 10 min. The amplified PCR products was loaded in 2% agarose gel prepared with 1X Tris-acetate-EDTA (TAE) buffer and stained by red safe DNA staining solution (GeNetBio, Korea). DNA ladder 100-bp (Genedirex, Taiwan) was utilized as a molecular size standard. An image was captured for data analysis.

Data analysis

An image with 22 wells representing all isolates of *C. pseudotuberculosis* was firstly recorded manually for the presence or absence of DNA bands in gel obtained from ERIC-PCR and then finally analyzed using the GelJ software version 2.0 (available at https://sourceforge.net/projects/gelj/) to generate dendrogram (Heras *et al*. 2015). The clustering of the isolates was performed based on Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) analysis and Dice similarity coefficient with a tolerance of about 1%. Isolates with a similarity coefficient equal to or above 90% (Similarity thresholds of ≥ 90%) were clustered as the same genotype (Taha 2021). Strains were clustered according to their genetic differences with in the same and the different animal species (Caprine and Ovine strains).

RESULTS AND DISCUSSION

The purpose of this study was to determine the level of genetic similarity and diversity among the 22 *C. pseudotuberculosis* strains from sheep and goats with CLA slaughtered in Duhok abattoir, to compare the efficacy of using sequence analysis of the *rpoB* gene (Abdulrahman 2021) with the ERIC PCR fingerprint (this study) for these used *C. pseudotuberculosis* strains and finally, is to find out the fact that is it possible that the strains isolated from sheep have the same genetic profile as the goat's strains.

According to the ERIC-PCR fingerprinting analysis (Fig. 1 and Fig. 2) and depending on the differences in the number and size of ERIC sequences found in each isolate, the results showed that the genetic similarity among 22 *C. pseudotuberculosis* isolates was between (55%-100%) and all isolates were grouped into13

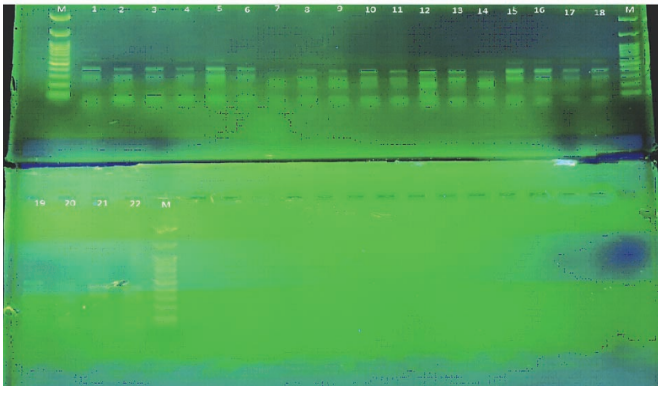

Fig. 1. ERIC-PCR DNA fingerprint patterns of 22 *C. pseudotuberculosis* strains isolated from pus material from sheep and goat with CLA. Lane M: 100 bp ladder, lane 1-22 test samples.

genotypes (1-13) according to 90% cut off similarity coefficient, in which genotypes 8, 9 and 12 represented the most prevalent clone and its variants among the isolates comprising (10/22; 45.4%) of total isolates. Four strains were clustered in genotype 8, followed by genotype 9 and 12 each with 3 strains. On the other hand, each of genotype 3 and 4 was consisted of 2 strains. The remaining genotypes (1, 2, 5, 6, 7, 10, 11 and 13) were included single strain only (Table 1, Fig. 2). This high level of genetic heterogeneity indicating that many clones of *C. pseudotuberculosis* circulate in this slaughter house, which could come from various geographic regions as this abattoir receive animal for slaughtering from different location (including both local and imported animals from the neighboring counties). Guimarães *et al*. (2011b) and Sellyei *et al*. (2017) have discovered that the entrance of animals from various locations may have supported in the dissemination of a large number of bacterial genotypes. In general, microbial genetic diversity is generated by point mutations or the insertion or deletion of specific DNA sequences. This will result in strain variations among microbial communities and this can occur following passage across various hosts or ecosystems (Jerome *et al*. 2011). This is a good explanation of why there is such genetic diversity seen among *C. pseudotuberculosis* strains in this study.

Despite the findings of this study, several studies have claimed that *C. pseudotuberculosis* is genetically stable (it has seldom undergone substantial genome reorganization and has preserved its ancestral genomic architecture), and this bacterium globally has a high level of genotypic similarity (Connor *et al*. 2007, Abdulrahman 2021). Nevertheless, the variety of clinical symptoms seen in sheep and goats with CLA, as well as the vaccines variable effectiveness, imply that more than one strain may circulate (Soares *et al*. 2013).

The strains of *C. pseudotuberculosis* that utilized in this investigation were previously thought to be clonal, with no genetic variation (all belonging to the same lineage), according to a prior study that used a molecular typing approach based on sequence evaluation of the *rpoB* gene (Abdulrahman 2021). However, in current study, all strains were clustered into 13 genotypes and high genetic diversity was established by using ERIC PCR technique. The reason behind the efficiency difference between both technique (ERIC-PCR technique and *rpoB* sequencing) is that the genetic diversity assessment by *rpoB* sequencing is depended on specific area of chromosome (Ogier *et al*. 2019).Therefore there is limited chance for the determination of genetic changes by *rpoB* gene sequence analysis. In contrast, ERIC primers are usually present in multiple loci of bacterial chromosome, so the whole chromosome can be assessed for any diversity in DNA sequence (there are multiple annealing sites for the ERIC primers on the bacterial genome and

Table 1. Genotypic pattern of 22 *C. pseudotuberculosis* strains isolated from pus material from sheep and goat with CLA.

Strain number	Animal species	Genotypic pattern
21	Sheep	Genotype 1
7	Sheep	Genotype 2
18	Sheep	Genotype 3
17	Sheep	Genotype 3
16	Sheep	Genotype 4
15	Sheep	Genotype 4
14	Sheep	Genotype 5
12	Sheep	Genotype 6
13	Sheep	Genotype 7
11	Sheep	Genotype 8
9	Sheep	Genotype 8
10	Sheep	Genotype 8
8	Sheep	Genotype 8
22	Sheep	Genotype 9
20	Sheep	Genotype 9
19	Sheep	Genotype 9
6	Sheep	Genotype 10
5	Sheep	Genotype 11
4	Goat	Genotype 12
3	Sheep	Genotype 12
2	Sheep	Genotype 12
1	Goat	Genotype 13

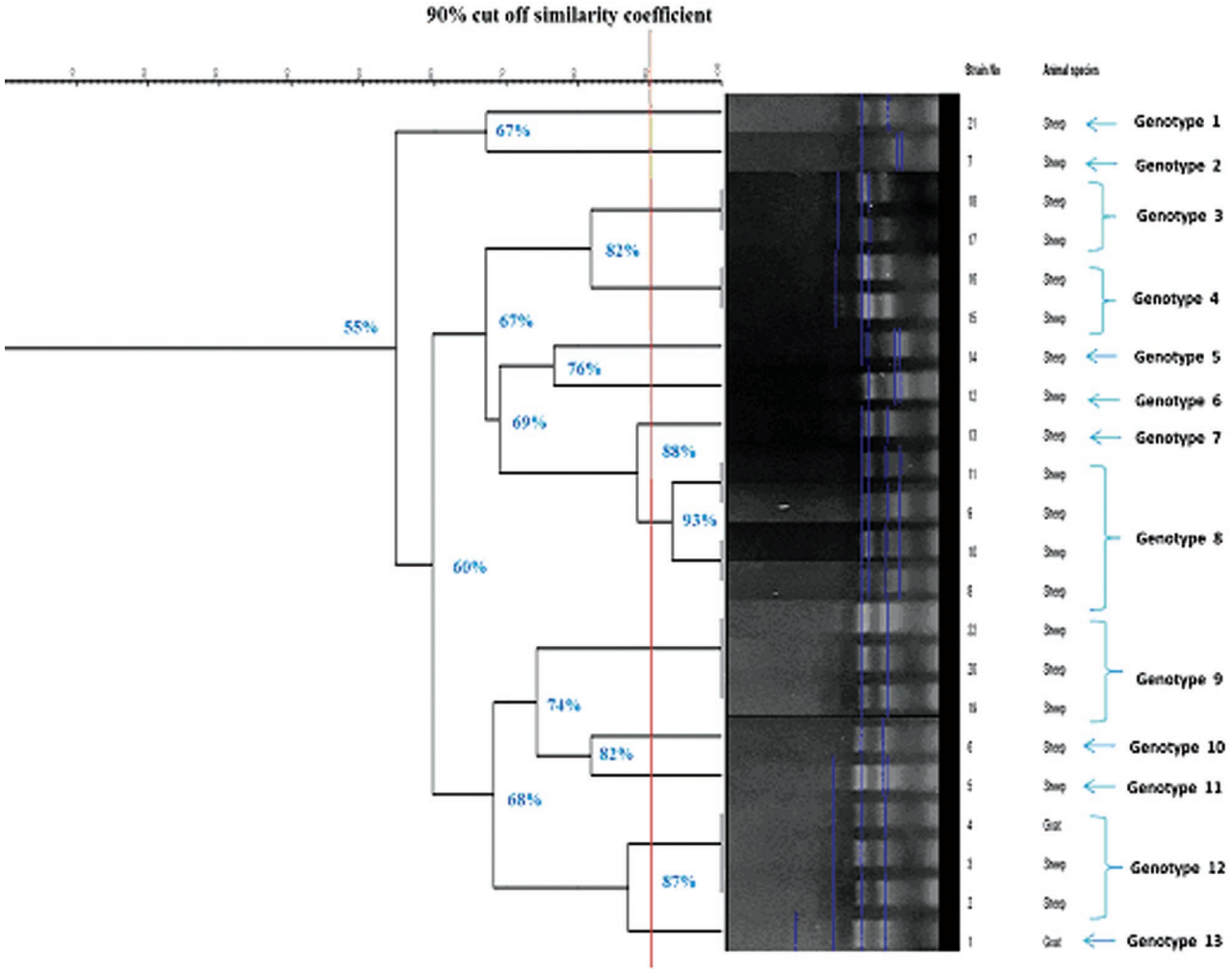

Fig. 2. Dendrogram generated from ERIC-PCR showing banding pattern of *C. pseudotuberculosis* strains isolated from pus material from sheep and goat with CLA.

different DNA bands sizes can be obtained after observing under gel documentation system) (Wilson and Sharp 2006) and this may give a maximum possibility to assess the genetic diversity using ERIC-PCR. This finding suggests that ERIC PCR, when compared to other molecular typing tools (ribotyping) is a useful genotyping technique for distinguishing strains in bacterial species, and the results of this study support the concept that ERIC PCR has a high discriminatory power and is an excellent molecular typing method for identifying genetic variation (tracing the source of infection) (Dorneles *et al.* 2012, Haas *et al*. 2017, Taha 2021).

Regarding the diversity and similarity of strains withinthe animal species, most strains (13/22; 59%) that were isolated from sheep were showed 100% genetic similarity.For example, strains number (17 and 18), (16 and 15), (11 and 9), (10 and 8), (22, 20 and 19), and (3 and 2).Whereas the two strains from goat were showed 87% of similarity (13% were genetically diverse) (Table 2, Fig. 2). Connor *et al.* (2007) have claimed that without *recBCD* genes (encoding of re-combinational repair enzymes), genome stability in *Corynebacteria* may be probable, as these genes are concerned with chromosomal inversion development. Similarly, Keesing *et al*. (2010) found that conserving intact ecosystems and associated endemic biodiversity may lower the incidence of bacterial infections (reduced biodiversity influences infectious disease transmission, resulting in reduced bacterial exchanges) among various hosts. The loss of biodiversity can have an impact on the spread of bacterial diseases by changing the population of hosts or vectors (Keesing *et al.* 2006). Another reason for this situation is that these sheep may be raised with in the same ecosystem (all came from the same geographic region). All of the above-

Table 2. Percentages of genetic similarity and diversity between some strains within the animal species (sheep and goat).

Animal species	Strain number	Percentage of genetic similarity
Sheep	21 and 7	67%
	18 and 17	100%
	16 and 15	100%
	14 and 12	76%
	13 with 11, 9, 10 and 8	88%
	11 and 9	100%
	10 and 8	100%
	22, 20 and 19	100%
	6 and 5	82%
	3 and 2	100%
Goat	4 with 1	87%
Sheep with goat	4 with 3 and 2	100%
	1 with 2, 3, and 4	87%

mentioned factors may be accountable for the genetic similarities of *C. pseudotuberculosis* strains in sheep.

About the diversity between both animal species, most strains (18/22; 82%) that were isolated from sheep showed a relative diversity to that of goat strains (Fig. 2). Signifying that there was little chance of cross-distribution of this bacterium between these two host species (Oliveira *et al*. 2014), as all of these strains may came from different regions and that the two animal species were separately raised (differences in their geographical distribution) (Dorneles *et al*. 2012), which could decrease the rate of exchange of *C. pseudotuberculosis* strains between these animals, with subsequent emergence of diversity. As previously mentioned, that this abattoir has an importation of animals from various countries and this has allowed the entry and spread of different *C. pseudotuberculosis* strains and based on above mentioned reasons, there was such diversity of strains between these two animal species.

CONCLUSION

Large numbers of *C. pseudotuberculosis* clone are circulating in our area which could come from animal importation from different countries. The obtained results also indicate that ERIC-PCR fingerprinting technique could be a useful tool for the screening of genetic differentiation, epidemiological relationships among bacterial isolates and establishing relationships between clinical isolates of *C. pseudotuberculosis*and sources of infectionand provides several benefits over other DNA-based genotyping techniques.

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