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Objectives: The present study aimed for isolation and identification of Corynebacterium species from ruminants. Antimicrobial sensitivity and detection for some virulence factors were done.

Methods: Two hundred and fifteen samples were gathered from various abscess locations in different species, inoculated on Baird Parker agar with 1% tween 80 then identified by biochemical tests. Antimicrobial sensitivity by MIC test was done with levofloxacin, Zinc oxide Nanoparticles, and Egyptian propolis extraction. Some isolates of Corynebacterium Pseudotuberculosis were identification of by 16Sr RNA PCR.

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Abstract- Background: Ruminants suffer from suppurative infections caused by the corynebacteriaceae affecting animals and humans. They cause severe economic losses and a high culling rate with poor treatment efficacy.

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Results: Positive suspected isolates were 22 isolates for *Corynebacterium* (C.) *pseudotuberculosis* whilst 5 isolates for *Trueperella* (T.) *pyogenes* as they grow as small, dark grey opaque colonies. C. *pseudotuberculosis* was positive for esculin, urease, catalase, Ellman, and Beta-D-Fucosidase while T. *pyogenes* was negative for them. All isolates showed phenotypic virulence factors such as hemolysin, and lipase enzymes with lecithinase activity, and biofilm formation. Antimicrobial sensitivity showed sensitivity to levofloxacin and resistance to the other while showing anti-biofilm formation at the same concentration. 8 isolates of C. *pseudotuberculosis* were sequenced and recorded in the gene bank with accession number ON899860.

Conclusions: This study refers to the high pathogenicity of *Corynebacterium* spp. and their risky resistance to the antimicrobials which need modern investigations for treatment.

1. INTRODUCTION

Ruminants are suffered from numerous kinds of infections caused by the corynebacteriaceae family. Corynebacteriaceae belong to the Actinomycetales order with many nomenclatural updates in recent years (Markey et al., 2013; Algammal, 2016). This genus belongs to the CMNR group (*Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus*), a heterogeneous group of pathogenic bacteria (Dworkin et al., 2006) characterized by diverse pyo- or granulomatous clinical infections that affect human and animals (Tauch and Sandbote, 2014; Thiago et al., 2021).

They are intracellular, cocco-bacilli, gram-positive microorganisms, non-capsulated, non-motile,

non-sporulating, and facultative anaerobes (Markey et al., 2013; Chandran et al., 2016; Juliana et al., 2019). Under the microscope, they are arranged in the form of palisades or similar to Chinese letters (Markey et al., 2013) or short coryneform rods (Park et al., 2022). *Corynebacteria* are pyogenic bacteria found externally on the skin, and mucous membranes and hidden internally in the intestinal tract of animals and humans (Bernard, 2012; Feßler and Schwarz, 2017; Park et al., 2022).

Some *Corynebacterium* species which have been detected in animals, documented to have zoonotic transmission as by the close contact with domestic or wild animals, i.e., during the occupational handling of animals, by animal bites, or by other means. Raw milk or dairy products without pasteurization often used for increase the risk of transmission of zoonotic pathogens as *Corynebacteriaceae* to humans (Langova et al., 2022) CLA has passive socio-economic effects that are chiefly defined in zoonosis, decrease the hide and market value, reproductive disorders and reduction of wool crop. It leads to dwindling in body weight, in wool and milk production even in the reproductive performance with elevation in the culling rate resulting to death of the infected animals with losses to the farm (Oreiby et al., 2014 and Burmayan and Brundage, 2021).

All ranchers in many countries suffer financial losses due to herd infection with *Corynebacteria* spp. as they cause numerous forms of pyogenic diseases (Dorella et al., 2006; Abd El Tawab et al., 2019) in addition to emaciation One of them is *Corynebacterium* (C.) *pseudotuberculosis* that is the main causative agent for Caseous lymphadenitis in Goat and sheep (Algammal, 2016; Thiago et al., 2021; Sting et al., 2022). 2- Mastitis in Cattle (Markey et al., 2013). 3- Ulcerative lymphangitis and ventral abscess in cattle (Markey et al., 2013; Vikas et al., 2017), Horses, and pigs (Markey et al., 2013). Also *Corynebacteria* spp. are reclassified into another genus including C. *pyogenes* as *Trueperella* (T.) *pyogenes* (Dworkin et al., 2006; Yassin et al., 2011; Magdalena et al., 2019). Conversely, T. *pyogenes* could cause a primary pathogen, infection usually follows a physical or microbial trauma that disseminates the organism (Rosenberg et al., 2014). T. *pyogenes* can cause numerous economically significant suppurative infections involving the skin and visceral organs including mastitis (Quinn et al., 2002; Unnerstad et al.,

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2009), pneumonia (Fulton et al., 2009), endometritis (Williams et al., 2005), liver abscess (Dore' et al., 2007), peritonitis and Pleurisy in sheep, goats, and cattle (Rosenberg et al., 2014; Abdallah, 2016). Many authors recorded the affected LNs illustrates marked enlargement with either thick creamy green pus with a central caseated core surrounded by dense fibrous capsule as (Torky et al., 2023).

This study aimed to discuss the conventional and the modern techniques to identify *Corynebacteria* spp. isolated from ruminants. Then further determination to some virulence factors and sensitivity to some antimicrobial agents would be done.

II. MATERIAL & AMP; METHODS

a) Ethical Approval

The research was carried out with the approval and in accordance with the guidelines of the local Ethics Committee at the Faculty of Veterinary Medicine, Benha University, Egypt. All the procedures in the study respect the ethical standards and the protocol was approved by the Ethics Committee with the code BUFVTM 12-10-22.

i. Samples Collections

One hundred ninety-seven swabs were taken from diseased farm animals (sheep, goat, cattle) and eighteen lesions from slaughtered one from different farms and slaughtered houses within Gharbiya & Menoufia Governorates from 8, 2021 till 4, 2022 (Table 1) (Fig. 2). Samples were taken as biological swabs from swollen lymph nodes or any superficial abscess and the whole affected lymph nodes from a slaughtered animal. The extraneous surface of the struck lymph node was cleaned, disinfected by povidone-iodine 7.5% then incised by a sterile blade. After that, the samples were taken from the inner surface and the pyogenic membrane after a gentle squeeze and were taken in a tube with brain heart infusion broth in an ice tank as quickly as possible.

Bacteriological examination according to (Markey et al., 2013, Zasada and Mosiej, 2018).

All collected samples were streaked in triplicate for each sample into plates of brain heart infusion (BHI) agar (Hi-media), 5% fresh heparinized sheep blood agar, and Baird Parker agar. All plates were supplemented by tween 80 by 1% for Lipophilic corynebacteria in addition of utilization of *C. pseudotuberculosis* (ATCC® 19410™) as control positive, then incubated for 24-48 h at 37°C. Suspected colonies were tested for color, motility, consistency, catalase, oxidase, and stained Gram's stain (EDM) then all colonies with Gram-positive forming cocco-bacilli, negative for oxidase and nitrate, and non-motile in semi-solid agar were selected, then tested for traditional biochemical tests, and VITEK test.

ii. Identification of *Corynebacterium* Species

Identification by vitek 2 compact system (biomerieux, 2006).

A sterile swab was used to transfer many colonies of fresh pure colonies to 3ml sterile saline in a clean plastic test tube. The turbidity should be calibrated by a turbidity meter called VITEK® 2 DensiCHEK™ then insert the ANC card in suspension in less than half an hour. The test tube with the inoculated card was loaded in a vacuum chamber station then the vacuum was applied and the micro-organism suspension was forced into micro-channels. The final data were obtained and automatically printed within 24 hrs.

Identification of some isolates of C. Pseudotuberculosis by PCR acc. to Pacheco et al., 2007).

By using Master Mix and Oligonucleotide primers sequences from Metabion (Germany). forward (ACCGCACTTTAGTGTGTGTG), reverse (TCTCTACG CCGATCTTGAT) at 816 bp with Cycling conditions: Primary denaturation (94°C 5min) 2nd denaturation (94°C 30sec), Annealing (58°C 40sec), Extension (72°C 50sec), Final extension (72°C 10 min) for 35cycles.

For sequencing reactions, 16Sr RNA PCR product of sample no.1 (540 bp length) was purified using QIAquick Gel Extraction Kit Protocol: (Qiagen Inc. Valencia CA and PCR Clean up sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/ Applied Biosystems, Foster City, CA), with Cat. No. 4336817. according to (Sanger et al. 1977) and the instruction of the manufacturer by Thermo Fisher Scientific, Schwerte, Germany. The sequences were edited and aligned using BioEdit version v7.0.9. Software A BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) was initially performed to establish sequence identity to GenBank accessions.

Detection of some phenotypic virulence factors:

b) Biofilm Formation

According to (Hassan et al., 2011)

All samples were tested by the tube and the congo red agar method for the detection of biofilm formation. Assessment of biofilm-forming quantitatively by using tube method and according to results lay down by. Bacteria are classified as weak, moderate, and strong biofilm producers. The strong Biofilm formation was detected using the CRA method, color of bacterial colonies was checked as black colonies with dry crystalline consistency indicating a positive result, weak slime producers usually remained pink. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result. The test was repeated three times with the reference

strain *Edwardsiella tarda* MW362142 as a positive control for biofilm formation (Abd El-Tawab et al., 2021).

Phenotypic virulence factors of Corynebacteria isolated according to (Yang and Fang, 2003, Markey et al., 2013) The hemolytic; amylase; proteolytic (caseinase); lipolytic and lecithinase activities for *Corynebacterium* Spp. isolated and CAMP test detection of the synergistic or the reverse hemolytic activity with *Staphylococcus aureus* ATCC® 6538.

c) Antimicrobial sensitivity test by Determination of minimum inhibitory

Concentration (MIC) method according to CLSI (2006) and The European Committee on Antimicrobial Susceptibility Testing (2019). ZnO-NPs and Egyptian propolis were obtained from the animal health research institute, El Doki, Egypt, and their concentrations were according to (Hegazi and Abd El Hady, 2002). ZnO-NPs was with a particle size of 371nm with 1.137OD wave length in nm and its cytotoxicity was IC₅₀ 100µg/ml on monkey kidney cell line assessed by SRB assay according to (Skehan et al., 1990). The MICs of antibiotics, Egyptian propolis, and ZnO-NPs against *Corynebacterium* isolates were determined by the broth micro-dilution method. The concentrations used in the MIC test were as follows; ZnO-NPs (100 to 10 µg/ml); Egyptian propolis (10mg/ml to 1,25mg/ml), Oxytetracycline (4 to 16µg/ml), penicillin G (25 to 8µg), levofloxacin (25- 8µg/ml).

The MIC of selected Antibiotics was defined as the lowest concentration of antibiotic that inhibits bacterial growth and no visible growth is observed as compared with both a positive control (culture broth containing bacteria only that should appear turbid) and a negative control (culture broth without bacteria that should remain clear). In addition, the half maximal inhibitory concentration (IC₅₀) of ZnO-NPs against *Corynebacterium* isolates was determined. The Experiment was performed in triplicate and quantitative assessment of biofilm formation on conge red agar.

III. RESULTS

a) Detection of *Corynebacterium* Species

Positive suspected *corynebacterium* species isolated were 27 from 215 samples with specific greenish colored pus (fig.3) as 19 out of 144 sheep, 5 out of 61 goats, and 3 out of 10 cattle by 12.56% of the total number.

Distribution of Corynebacterium species isolated from abscess locations as table (2).

Out of 19 strucked sheep, the distribution of *C. pseudotuberculosis* was 9 males (5 of them were old and 4 were from dead animals), 1 parotid lymph node (11.1%), 1 fatty tail (11.1%) and 3 pre-scapular lymph node (33.3%) and 10 females (5 of them were old and 5 were young) as 2 sub-mandibular lymph nodes (20%), 2 parotid lymph node (20%) and 2 pre-scapular lymph

node (20%), 1 retro-pharyngeal lymph node (10%), 2 pre-femoral lymph node (20%) and 1 in the fatty tail (10%). However, the distribution of *C. pseudotuberculosis* in 3 strucked goats, was 2 males (1 young and the other was old) by 1 in pre-femoral lymph node (50%) and 1 in sub-mandibular lymph nodes (50%) whilst 1 young female by 1 retro-pharyngeal lymph node (100%). We found *T. pyogenes* in cattle in 3 cows with abdominal abscess (100%) while we found it in two lactating does as non-specific infection in pre-scapular lymph node (100%). These results revealed many points: Sheep was the most affected species mainly after manual shearing by scissors in fixed breeding. The main affected L.N in sheep was in the head region- due to fighting- despite it having fewer affections in goats and being absent in cattle.

b) Cultural and Biochemical Characteristics of Isolates

All *Corynebacterium* species isolated were Gram-positive non (sporulated–capsulated–motile) cocco-bacilli to short-chain bacilli which can appear in pairs or single arrangements. The most characteristic club shape was in form of acute angles or Chinese-like appearance as in fig. 4. After 48 h of incubation, their colonies appear as minute, white, smooth, dry colonies on BHI agar surrounded by a thin zone of β hemolysis on 5% fresh heparinized sheep blood agar. The colonies were waxy and splashing with flame with hard collecting as they were swaying on the agar surface. On Egg-yolk Tellurite or Baird Parker agar, the colonies were small, dark grey, and opaque in appearance after 24-48 h (fig. 5). All tested isolates were non-motile, negative for oxidase, and nitrate reduction tests whilst 22 isolates were positive for Urease and catalase tests and 5 isolates were negative for them.

Identification of Corynebacterium species by vitek 2 compact system. As in fig. 6 & 7 *C. pseudotuberculosis* was positive for esculin, urease, catalase, Ellman and Beta-D-Fucosidase whilst *T. pyogenes* was negative for all of them.

i. The result of 16Sr RNA of *C. pseudotuberculosis*

Out of identified *C. pseudotuberculosis*, 8 selected isolates were tested for 16Sr RNA and all were positive at 816 bp as in (fig. 8). The sequence of 16Sr RNA has accession number ON899860 partial sequence and its length 539 BP (780 BP), has similarity with 31 accession number in gene bank with 99.49% as in (fig. 9).

Fig. 8: Agarose gel electrophoresis of PCR for amplification products of The 16S ribosomal RNA gene (16S rRNA) for 8 *C. pseudotuberculosis* isolates. All Lanes show positive amplification of 16S rRNA gene at 816 bp. Lane L: DNA ladder at 100-1000bp. N.: Negative control (sterile DNase/ RNase free DEPC water). P.: Positive control (*C. pseudotuberculosis* (ATCC® 19410 TM)).

ii. The Results of Phenotypic Virulence Factors

All isolates had hemolytic, lipolytic, proteolytic (caseinase), and Lecithinase activities. The CAMP test justifies that *C. pseudotuberculosis* inhibits the staphylococcal beta-hemolysin reaction (reverse CAMP test) whilst *T. pyogenes* enhances it (pos. CAMP-like reaction). Addition to 90% of isolates produces biofilm as shown in Tables (3, 4) and fig.10 & amp;11.

c) The Result of Antimicrobial Sensitivity Tests

All isolates were resistant to all tested kinds of anti-microbial except levofloxacin at 8ug/ml. But nano zinc oxide 100ug/ml and propolis extraction at 10mg/ml can eradicate the biofilm formation of *C. pseudotuberculosis* (fig 12.).

IV. DISCUSSION

The pyogenic bacteria are ferocious and annoying bacteria for animals and ranchers. Not also it affects animal health, but also causes financial losses for the ranchers. One of them is *C. pseudotuberculosis* which causes Caseous lymphadenitis (CLA) in sheep and goats. CLA is a zoonotic, contagious, and chronic bacterial disease which causes granulomas in lymph nodes (superficial and visceral) extending to several internal organs [Banke et al., 2021; Burmayan and Brundage, 2021; Santos et al., 2021].

Another one is *T. pyogenes* which causes various suppurative infections in domestic animals as mastitis, abscesses, pneumonia, and lymphadenitis as in (Ribeiro et al., 2015; Rogovskyy et al., 2018). In this research, the prevalence of *C. pseudotuberculosis* isolated from alive and dead animals was in a congenial ratio with the result reported by (Oreiby et al., 2015; Abd El Tawab et al., 2019, and Torky et al., 2023). Conversely, it is a minor ratio compared with the one reported by (Al-Gaabary et al., 2009). In in-contrast that it is a major ratio reported in (Al-Gaabary et al., 2013;

Al-Gaabary et al., 2015). The prevalence of *T. pyogenes* was reported in cattle and goats as reported in (Abdallah, 2016; Rogovskyy et al., 2018) with different ratios. Accurate identification of CLA relies on PCR detection methods such as 16S rRNA gene as reported in Public Health England (2014) and Algammal, 2016 that was present in all tested isolates. The molecular genetic sequence analysis of 16S rRNA gene has facilitated a much tighter circumscription of the genus *Corynebacterium*. The availability of comparative 16Sr RNA gene sequence improved phenotypic data has resulted in much improved and more reliable species identification Public Health England (2014).

The result of MIC test showed the sensitive of *C. pseudotuberculosis* to levafloxacin and resistance to pencilline which agree with Torky et al., 2023 and dis agree with Markey et al., 2013 recorded its sensitivity to penicillin. The propolis and ZnO-NPs exhibited resistance from tested *C. pseudotuberculosis* but they

have a good anti-biofilm activity against it as reported by (Santos et al., 2021 and Abdelghafar et al., 2022). That may be due to their unreasonable use of them during the rearing period. Subsequently, it was a necessity to detection of their ability for biofilm formation as there is a close connection between them. From this study, most infections occur in fixed and mixed breeding, especially after the shearing season as reported by (Oreiby et al., 2014). The sensitivity and specificity for biofilm formation were much more accurate by the tube method than CRA which was confirmed by (Hassan et al., 2011).

V. CONCLUSION

Corynebacteria cause obstinate infections with unidentified rules for treatment. CLA leads to a dwindling in body weight, wool, and milk production even in the reproductive performance with an elevation in the culling rate resulting in losses to the farm. Further modern researches should be done to know how to handle this microorganism.

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Conflict of Interest

The authors declared no conflict of interest.

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- نم قلو زعمال بذالك لسل اي ريكيب يل ع فرعتا (عقيد قلدول - عبالا عقيدول) تارتجمل دب ع ضوع فرشا ، * 1 ميلس رمع ينام ، 2 ناميلس عي 2 باوتال
- شوحبال زكرم - تاي رطفالو عانملاو ايرتكلبال مسق 1- رصم ، اهنب عرف - ناويحل عحص شوحب دهعم - عي عارزل بطل عي لك - تاي رطفالو عانملاو ايرتكلبال مسق 2- : عصالخل رصم - اهنب ععماج - ي رطيبال
- اهببست عدي عودع نم تارتجمل يناع : عي فلخل بيصت ععونتم تاحي قتب زيمنت يتل تاي دتول رئاسخ ببست اهنأ امك . يرخلأ تاناويحلوال ناسنالا عبالا لدعم عافت رل عحدا عي داصتقا ماعلال
- ايري تكبال عاونأ لز ع وه انقي قحت نم دصقل : فادهال زاهجب اهراب تخا مت يتل تارتجمل نع عي دتول اعصقت سال نم ديزم يل عفاضالاب . (Vitek 2) 2 كتيافل تابوركيمل تاداضمل اهتيساسح نع دي دحتو ملي فويبال ليكشتو ، CAMP رابتخاو يري م يل وبل لسل ستمل رابتخا PCR
- عاونأ نم عني ع رشع قس مخو ني تئام عمع مت : عي رطل قيرط نع عي هتق اهديدحت مت مت عفل تخم تارابتخال عارجا مت . ركراب دريب راجا يل ع اهت عارز هبتشمل فئاوطل عي مجل عيديل قتل عي ئاي ميكيو يبال مت مت . مارج عغبص عطساوب اهراب تخا مت يتل اهي فطوغضمل Vitek 2 ماطن عطساوب اهفاشك 16 س يسابي رل انرل لسل ست متو (biomerieux ، 2006)
- سزول كريبوي تودوس ميري تكاب يني روكلل رابتخا عطساوب تابوركيمل تاداضم عي ساسح عارجا مت ني ساسكول فوفيلل م ادخت ساب MIC سيل و بوب رل جارختساو كنزل ديسكال عيونا نل تامي سجالو يري صمل
- نم قلزع 22 عي باجي اهب هبتشمل تالزعل تنك : جيئاتنل عي دتول نم تالزع 5 و عبالا عي دتول ريغ عري عص تارمع تسم لكش يل ع ومنم ان شيح عي قحتل تنك . قماغ يدامر نول تاذ عفاش زايرويلاو نيلوكسي لال بلجوم بذالك لسل ضرر ايرتكل زادي سوكوف-يدي-اتي بو ناملي او زالتاكالو
- مل عيسنل اب عي بل س عي قمل ايري بورت تنك امنيب لثم عي طمن قوارض لم او ع تالزعل عي م ع ترعظا . عيوي حل عيشال نيوكسو ، زابيلل ميذن ، نيزيلوميل تابوركيمل تاداضم عي ساسح ترعظا راعظا عم يقيابلل عموامو نيساسكول فوفيلل مارجت عي ساسح 8 . زيكرتل سرفنب ملي فويبلل داضم نيوكسو 16Sr RNA ل قبحوم تنك عبالا عي دتول ايرتكل نم تالزع كن يفل عجل لسل ستمل بناجب ON899860 ل عدمل مقرب تانيجل عفاضالاب تاي دتول ررض جيئاتنل مده : تاجاتنت سال قري طخل امتوامم ناف ، لذل يل عي دتو تاقيقحت يل عراجب انل عجت تابوركيمل تاداضم جال علل
- ع- عي قمل عي دتول - عبالا عي دتول : عي سيئرل تامللكل عيوي حل عيشال نيوكسو - VITEK- 16S r RNA.