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Apoptosis of somatic cells caused by *corynebacterium bovis* isolated from mastitic cows in Egyptian farms.

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Characterization and identification of *Corynebacterium bovis* (*C.bovis*) and determination its contagious effect when were subjected to in vitro apoptotic assay .A number of 412 pooled milk samples were collected out from mastitic cows that were included in a field survey to identify subclinical mastitis (SCM) using the California Mastitis Test (CMT) in different regions in Egypt and bacteriological investigation for *C.bovis* existence. The *C.bovis* isolates were biochemically identified by Analytical profile index (API) and molecularly detected by Polymerase Chain Reaction (PCR) technique following by sequencing of 16 Sr.RNA gene. The apoptotic effect of *C.bovis* was investigated in vitro by comet assay. The present study reported that the percent of (*C. bovis*) from 412 milk samples was17 (60.7%) samples out of 28 isolates of *Corynebacterium spp*. Irregular Gram-positive bacilli were isolated in pure cultures and API-Coryne System 3.0 was used to identify all the milk isolates infected with *C. bovis* (code number 4501014; 99.9% confidence level). The PCR test and 16 Sr.RNA sequencing gene confirmed *C.bovis* strain. Furthermore, the comet assay revealed that *C. bovis* had a strong effect on somatic cell's DNA integrity .*C. bovis* is relevant in the pathogenesis of bovine mastitis. Moreover contagious bacteria represented by *C. bovis* had the stronger effect on the integrity of SCs DNA.

Keywords: ***Corynebacterium bovis,*** **Mastitis, Sequencing of Genes,16Sr.RNA, comet assay.**

INTRODUCTION

Bovine mastitis represents one of the most economically paramount diseases that affects on dairy cows. Bovine mastitic cows are frequently infected with Coryne form bacteria and lipophilic *Corynebacterium bovis* represents the most common species of this group **(**Weber et al., 2012**)**. During the last few decades, various strategies were applied to control mastitis. This resulted in an observed diminish in the clinical and subclinical mastitis cases caused by major pathogens. However, the subclinical mastitis caused by minor pathogens is still uncommon for dairy farmers (Taponen, and Pyorala 2009).*Corynebacterium bovis* represents one of such minor pathogens that frequently cause subclinical mastitis **(**Schuk 2009). The effects of *Corynebacterium* spp. on the production and composition of milk remain mostly unknown **(**Juliano et al., 2014).

The aim of this study is to identify and isolate *C. bovis* using API test, RCR technique and 16 Sr.RNA sequencing and evaluate the apoptotic impact of *C. bovis* on DNA integrity of somatic cells in vitro.

MATERIALS AND METHODS

California Mastitis Test (CMT):

Quarter milk samples were screened in the field using the California Mastitis Test (CMT) .Before samples collection; the udders were thoroughly disinfected with 70% ethanol and dried. The first strips were discarded and a milk sample from each quarter was tested by CMT. Milk samples with positive CMT reaction (+ or more)(Quinn et al.,2011), and positive for bacterial growth were classified as subclinical mastitis and clinical mastitis infected quarters CMT-positive quarters' milk samples were collected under aseptic conditions in labeled sterile screw caped bottles and kept at 4ºC for further lab investigations**.**

Animals and Sampling:

In this study, atotal of 412 pooled milk samples collected out from subclinical mastitic cows gave positive results of CMT in and around El Behera, El Qalubia, El Sharkia, Benisuef and El Menoufia Governorates of Egypt. Mastitic milk samples were collected during period from June 2015 till November 2016. All collected sample were transported directly in ice box to bacteriological laboratory of Animal Reproduction Research Institute in Giza Governorate.

Microbiological Culture Conditions

The isolation of causative organism *Corynebacterium spp.* in collected milk samples was done by spreading 10 µl of milk over 5% bovine blood agar plate. Corynebacterium-like colonies were initially identified according to Gram staining, colonial morphology, pigmentation and hemolytic properties. Colonies of irregular Gram-positive rods (IGPR) were re-cultured in brain heart infusion (BHI) added of 1% tween-80 and incubated for 24h. Then, the microorganisms were subjected to phenotypic and genotypic tests for identification as described below **(**Juliano et al., 2014).

Phenotypic Tests for *Corynebacterim bovis* Identification.

Microorganisms were characterized according to the commercially available semi-automated identification API-Coryne System 3.0 (bioMerieux, Lyon, France) under the manufacturer's instructions procedure according to **(**Langoni et al., 2016)**.**Decoded by the API web system (https://apiweb.biomerieux.com).

Gene Amplification and Sequencing.

Chromosomal DNA was extracted using a rapid boiling procedure according to **(**Reischl et al., 1994)., Detailed sequences of primers and cycling protocols were depicted in Table (1). PCR products were electrophoresed in 1.5% agarose gel containing 0.5X TBEat 100 volts for 40 min. Gels were stained by ethidium bromide and visualized by UV trans-illuminator.

A purified PCR product for 16S rRNA gene was sequenced directions on an Applied Bio systems 3130 automated DNA Sequencer (ABI, 3130, USA). Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit.

A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNA Star software Pair wise, which was designed by (Thompson et al ., 1994).And Phylogenetic analyses were done using maximum likelihood, 3505eighbor joining and maximum parsimony in MEGA6 **(**Tamura et al., 2013)

Culture of somatic cells.

In vitro culture of SCs was applied according to **(**Boutet et al., 2004). with some modification according to (Abeer et al., 2016).The SCs isolated from milk samples from healthy pooled, California mastitis test (CMT) negative,were grouped into:

Group 1:

Normal SCs

Group 2:

SCs + *In vitro* infected with C.bovis 0.5 Mac Ferland Somatic cells of each group was cultured into 96-well tissue culture plates (Nunclon Delta, Nunc, Roskilde, Denmark) contained RPMI 1640 medium (Sigma, MO, USA) supplemented with 1% glutamine, 10% fetal calf serum, 50*µ*g/ml streptomycin and 50 IU/ml penicillin (200*µ*l/well). The plates were incubated in humid chamber at 37°C for 3 days before analysis. After the incubation period, the pellets of the two groups of SCs were collected by centrifugation in cooling centrifuge for further investigations.

Comet assay.

The pellets containing somatic cells of each group was examined to evaluate apoptosis by detecting DNA damage using comet assay. Somatic cell's DNA damage was determined according to (Singh et al., 1988). About 100 randomly selected nuclei were photographed and

scanned for the detection of tail length, the percentage of DNA in the tail.

Statistical analysis

Differences between groups were analyzed according to (Bailey, 1995) by using one way analysis of variance (ANOVA), followed by Duncan multiple comparisons test using SPSS package version "20" for Windows. Values are represented as mean ± S.E. and $p < 0.05$ was considered statistically significant, $p < 0.01$ was considered highly significant and $p < 0.001$ was considered very highly significant.

RESULTS AND DISCUSSION

Of 412 pooled milk samples were collected out from mastiticcows, With respect to the molecular studies, the PCR analysis of 16S r.RNA gene using its specific primer sequence confirmed the a genus of *Corynebacterium Spp.* and species specific primer sequence for 16S r.RNA gene confirmed the C.bovis detected in 17 (60.7%) samples out of 28 isolates of *Corynebacterium spp*. Similar results were reported previously by (Juliano et al., 2014).They identified 180 *Corynebacterium* spp. isolates using 16S r.RNA gene, 167 (92.77%) isolates of which were *C. bovis.*

The sequencing data of 16S r.RNA gene indicated that. The gene sequences of 16S r.RNA were deposited in Gene Bank under the numbers HQ876172, JX298782, JX298784, JX298786, JX298788 and NR_118465**.**samples had similarity superior to ≥ 98% for 16S r.RNA gene only with the species *C. bovis* when compared to GenBank1 Library Reference online data. These investigations keep in line with (Watts et al.,2000).Theyreported that all sequences obtained from the 16S r.RNA gene that were analyzed with GenBank1 Library Reference online data were identified at the *Corynebacterium bovis* species level when their similarities to reference sequences were ≥ 98%.

The Phylogenetic tree based on the neighbor-joining method using 16S r.RNA gene sequences for different strains of *C. bovis* illustrated the similarity values obtained in the analysis of 16S r.RNA gene between strains(fig 1). In the same way (Langoni et al.,2016). Demonstrated that phylogenetic analysis of the clinical isolates belonged to *C. bovis* species had the similarity values obtained in the analysis of 16S r.RNA gene.

Our results reported that somatic cell's tail length and DNA% in tail induced by *Corynebacterium bovis* reached to its highest significant elevation at compared to normal control group. The comet assay can be used to assess genetic damage, but heterogeneity in the length of the tails was frequently observed (fig 2). These findings are in agreement with (Tharwat et al., 2011). There was an accelerated rate of DNA damage of PMN in diseased animals when compared with control cows.

Apoptosis that occurred in SCs was affected by bacteria that infected the mammary gland. Furthermore contagious bacteria represented by *Corynebacterium bovis* in the *in vitro* apoptotic assay had the stronger effect on SCs DNA integrity and DNA-fragmentation (table 2).

From the aims of this study were to evaluate genetic damage and heterogeneity occurred to SCs in response to *C.bovis* bacterial invasion.

Table 1: Primer sequences, target genes and cycling profiles of PCR assays used in this study.

Primer name Specificity	Sequence (5'–3')	Amplified product	PCR program	Refrance
16SrRNA C.spp	F-GCGAACGGGTGAGTAACACG R- TCTGCGATTACTAGCGACTCCG	1250 bp	95ºC/30s 58ºC/30s 72 ºC/90 s 35 cycles	Huxley et al.,2004
16SrRNA *C.bovis*	F-5'GCGAACGGGTGAGTAACACG3' R- 5'TCTGCGATTACTAGCGACTCCG3'	383 bp	95ºC/30s 60ºC/30s 72 ºC/45 s 35 cycles	Andreas et al.,2012

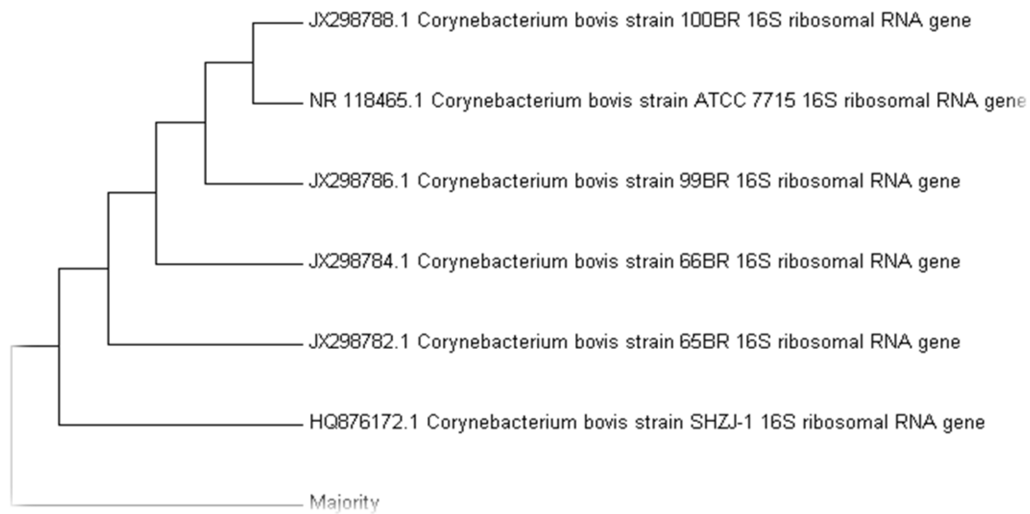

Figure (1): Phylogenetic analysis unambiguously demonstrated that the clinical isolates belonged to *C. bovis* species.

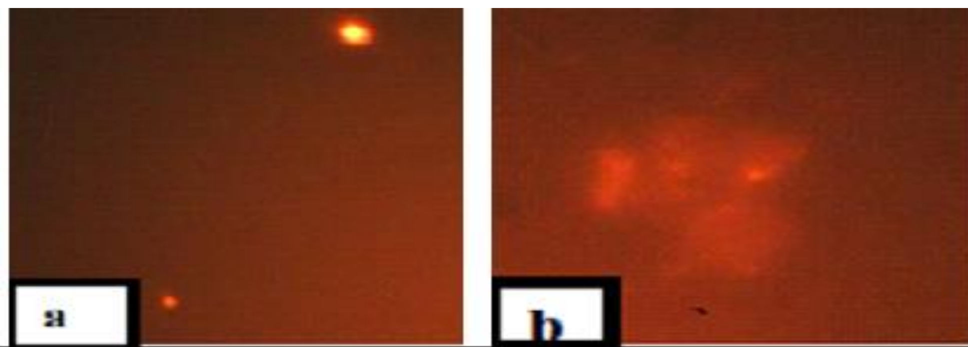

Figure (2): Comet assay of a- normal somatic cells group, b- somatic cells infected with *C. bovis* group. The cells were examined by fluorescence microscopy, fluorescent structures corresponding to the PI–stained nuclear DNA of the PMN cells.

Table (2):DNA damage , DNA% in tail and Tail length percent of normal, infected somatic cells groups. by using comet assay (Mean ± SD).

Groups.	DNA damage	DNA% in tail	Tail length	ANOVA
SCs	14.20±0.24[a]	5.04±0.20[a]	5.20±0.22[a]	**P-Value =0.000**
SCs +*C. bovis*	25.98±0.77[b]	21.22±0.49[b]	20.84±0.49[b]	**P-Value =0.000**

*SCs=Normal somatic cells, *C. bovis*= *Corynebacterium bovis.*
*Values are expressed as Mean ± SD of 5 bacterial isolates per group.
*Values that share the same letter at the same Colum are not significant.
* Values that share different letters at the same Colum are significant.

CONCLUSION

The present study has shown that mastitis particularly is a widely prevalent disease in the dairy farms of governorates Egypt at cow-level.
Moreover apoptosis in SCs was affected by Contagious bacteria represented by *C.bovis* had the stronger effect on the integrity of SCs DNA. It is also supported the differentiation and the diagnosis of *Corynebacterium bovis* must be grounded in well conducted studies that meet the assessment criteria related to milk production by the affected quarters.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

By Nabih, A.M carried out sampling and laboratory analysis. Wael, S. Abdel-Mageed, and Abeer ,M.ElSayed analyzed and interpreted the data while, Khalil ,Halfawy did the overall monitoring of the experiment and preparation of the manuscript. All authors have read and approved the final manuscript. All authors read and approved the final manuscript.

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