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Molecular characterization of snuffle-causing bacterial pathogens in rabbits in terms of prevalence and pathogenicity

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ABSTRACT

Respiratory disease, or snuffling, is a major problem associated with serious economic losses in the rabbit industry. The disease in rabbits is a polybacterial infection in nature, resulting from co-infection with more than one bacterial pathogen. To determine the involved bacterial pathogens in respiratory infections in terms of prevalence and pathogenicity, different specimens from rabbits with typical respiratory disorders are subjected to bacteriological examination followed by molecular verification through RT-PCR or PCR then sequencing of the PCR-product. The results identified Mycoplasma sp. as a major partner in mixed infection with virulent strains of *P. multocida* or *S. aureus*, suggesting that, initial infection with Mycoplasma sp. creates vulnerability to subsequent infection with *P. multocida* or *S. aureus*. Gross and microscopic analysis were also applied to demonstrate lesions accompanied by the detected bacterial pathogens. This study described the prevalence and characteristics of respiratory illness in rabbits domesticated in Al-Sharkia Governorate, Egypt and highlighted for the first time the role of Mycoplasma sp. in increasing the severity of rabbit-respiratory infections.

Keywords: Rabbits, Mycoplasma sp., *P. multocida, S. aureus*, Histopathology, RT- PCR sequencing

INTRODUCTION

Domestic rabbits are highly susceptible to developing gastrointestinal and respiratory illnesses when they are exposed to stress or extreme environmental conditions. Respiratory diseases in rabbits are second only to gastrointestinal diseases in importance, because they badly impact the rabbit industry and cause considerable economic losses (Soriano *et al*., 2012).

Respiratory illness in rabbits is known as snuffles, and it is recognized as a polybacterial infection caused by a combination of *Pasteurella multocida* with additional bacteria such as *Bordetella brochiseptica*, *Staph*. sp., *Pseudomonas* sp., *Chlamydia* sp., *Acinetobacter* sp., *Moraxella catarrhalis* and *Mycoplasma* sp., etc (Esther, 2003). *Pasteurella multocida* is one of the most frequently isolated pathogens from rabbits showing respiratory symptoms. It mainly causes upper respiratory illness (snuffles). The primary clinical symptoms of snuffles include rhinitis, sinusitis, otitis media, conjunctivitis and orchitis (Tayeb *et al.,* 2004). Primary respiratory signs usually develop into pneumonia, septicemia and abscess in lung, because *Pasteurella multocida* may spread hematologically, causing acute generalized disease, fever, and sudden death. Pathological investigation may also reveal pleuropneumonia, and septicemic congestion, petechiation and microscopic abscesses throughout the viscera (Barbara and Digiacomo,2000). *S. aureus* is also frequently recovered from pneumonic rabbits. Induced pathogenicity usually depends on host susceptibility and bacterial virulence, where it produces lethal toxin to kill the rabbit neutrophils and suppress the host defense mechanism. A virulent strain of S. aureus causes mastitis, pododermatitis, and subcutaneous abscesses at rabbit flock level, while infections with low virulence strains are restricted to individual rabbits (Vancraeynest *et al*., 2006).

Mycoplasma infection is usually asymptomatic and pathogenesis also vary according to the host and environmental factors (Schoeb *et al*., 1993), including co-infection with additional pathogens (Schunk *et al*., 1995). *Mycoplasma* sp. especially *M. pulomins* infect rat and mice causing snuffling in rats and "chattering" in mice (National Research Council, 1991). In rabbits, *Mycoplasma* infection is very rare; however, Barbara (2004) isolated *M. pulmonis* from nares and oropharynx of New Zealand white rabbits showing upper respiratory disorders. It is also previously documented the involvement of *Mycoplasma* in rabbit respiratory diseases. Reporting exudative bronchopneumonia and extensive foci of coagulative necrosis surrounded by inflammatory cells in naturally infected lungs with *Mycoplasma* while those cases of experimentally infected lungs revealed suppurative bronchiolitis and varying degrees of peribronchiolar mononuclear cell cuffing (Rodríguez *et al*., 1996).

The current study used multiple diagnostic approaches to determine the bacterial pathogens implicated in rabbit respiratory difficulties, as well as the most prevalent bacterial combinations of mixed infections in terms of prevalence and toxicity. It

reports the role of *Mycoplasma* sp. in the development of severe respiratory illness in domestic rabbits in conjunction with either *P. multocida* or *S. aureus*.

MATERIALS AND METHODS

Sampling:

A total of 80 samples were collected from different aged and diseased rabbits from Al-Sharkia Governorate farms; 30 nasal swabs from rabbits suffering from respiratory symptoms and 50 tracheas and lungs from freshly dead ones for bacteriological examination under complete aseptic conditions. Specimens from the tracheas and lungs of the diseased rabbits, either sacrificed or freshly dead, were collected after PM examination and fixed in 10% buffered neutral formalin. Paraffin sections of 2–3-micron thickness was prepared and stained with hematoxylin and eosin stain, then examined microscopically (Survarna *et al*., 2013).

Bacteriological culturing:

Collected samples were inoculated on 5% sheep blood agar, MacConkey agar and mannitol salt agar then incubated at 37°C for 24h for *P. multocida* and *S. aureus* isolation. Suspected colonies were initially identified by colonial morphology and Gram-staining as described by Weisburg *et al*. (1991) and Townsend *et al*. (1998).

Samples were also cultured according to Frey *et al*. (1968) for the presence of the fried egg colonies of *Mycoplasma* sp. *Mycoplasma* isolates identification was carried out by digitonin sensitivity test (growth inhibition) as described by Erno and Stipkovits (1973) and Razin *et al*. (1998). Biochemical characterization of purified *Mycoplasma* isolates including; glucose fermentation, arginine deamination and film & spot formation tests were done according to Fabricant and Freundt (1967) and Watson *et al*. (1988).

Molecular identification:

Extraction of genomic DNA:

DNA extraction of samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH, Catalogue no.51304) following the manufacturer's recommendations.

Primers sequences and PCR amplification:

The forward primer GPO-3 (5'-GGGAGCAAACAGGATTAGATACCCT-3') and reverse primer MGSO (5'-TGCACCATCTGTCACTCTGTTAACCTC-3') are part of the mycoplasma group-specific primer set. The thermal profile of the Mycoplasma 16SrRNA gene comprised of 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes (Van Kuppeveld *et al*., 1994).

All PCR reactions of *Mycoplasma* isolates were performed in a "Gradient Thermal cycler 1000S" (Bio – RAD, USA). The reaction mixture of total volume of 50µl; was 25µl Dream Taq Green PCR Master Mix (Thermo Scientific Company, Lithuania), 5µl target DNA, 2µl of each primers (containing 10pmole/µl) and the mixture was completed by RNAse DNAse free sterile distilled water to 50µl. The amplified PCR products were confirmed by using a 1.5% agarose gel, followed by UV visualization after ethidium bromide staining.

Purification and sequencing of *Mycoplasma* isolate:

GATC Company sequenced the PCR product using an ABI 3730xl DNA sequencer and forward and reverse primers. MegAlign, DNASTAR, Lasergene®, Version 7.1.0, USA, and BioEdit sequence alignment editor (Hall, 1999). MegAlign was used to rebuild the phylogenetic tree of sequences using the neighbor-joining approach based on ClustalW. (Thomposon et al., 1994). MegAlign computed the sequence divergence and identity percent.

Real-time PCR identification:

SYBR Green real-time PCR amplification:

All real-time PCR tests were done at Biotechnology Department, Zagazig Province lab, AHRI. Two µl of the extracted DNA was added to 18µl mastermix (iQ™ SYBR Green Supermix; Bio-Rad, USA). The mastermix contains: 10µl iQ™ SYBR Green Supermix, 2µl forward and reverse primers and 6µl deionized water. The following cycling parameters were used: one cycle of 95°C for 5 min, 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The fluorescence intensity of SYBR Green and the melting curve analysis were evaluated and a threshold cycle (Ct) under 35 and a specific melting temperature (Tm) indicated a positive result. Primers used in SYBR Green real-time PCR are shown in **Table (1)**

Table 1. Primers used for SYBR Green real-time PCR assays

Target gene	Sequence (5'-3')	References
***P. multocida* (*sodC*)**	AGTTAGTAGCGGGGTTGGCA TGGTGCTGGGTGATCATCATG	Ewers *et al*. (2006)
***P. multocida* (*hgbA*)**	TGGCGGATAGTCATCAAG CCAAAGAACCACTACCCA	Ewers *et al*. (2006)
***P. multocida* (*nanH*)**	GAATATTTGGGCGGCAACA TTCTCGCCCTGTCATCACT	Ewers *et al*. (2006)
***S. aureus* (*16SrRNA*)**	CCTATAAGACTGGGATAACTTCGGG CTTTGAGTTTCAACCTTGCGGTCG	Mason *et al*. (2001)
***Staphylococcus* (*ica*)**	CCTAACTAACGAAAG GTAG AAGATATAGCGATAAGTGC	Kumar *et al*. (2009)
***Staphylococcus* (*spa*) (X-region)**	CAAGCACCAAAAGAGGAA CACCAGGTTTAACGACAT	**Paniagua- Contreras *et al*. (2012)**

Taqman Real-Time PCR amplification:

All real-time Taqman PCRs were carried out in a total volume of 20µl with 6µl DNA template, 10µl master mix (Sensifast Probe No. Rox. Catalogue no. Bio-86050), 0.8µl of each primer, and 0.6µl of the probe(s) then the volume is completed to 20µl using deionized water. qPCR was performed on one step real-time PCR system (Thermo Fisher Scientific, Germany). The following cycling parameters were used: one cycle of 95°C for 5min, 40 cycles of 95°C for 10sec and 60°C for 50sec. Cycle threshold (Ct) for each sample were calculated in comparison to positive and negative controls and the parameters of analysis were as follows: exclude early cycle=7, minimum relative amplifications=0, and minimum amplification quality=5. Primers and probes were purchased from Sigma-Aldrich, St. Louis, MO, USA. Primers and probes were illustrated in **Table (2)**.

Regarding the *Pasteurella* isolates, amplification conditions were adjusted for the amplification of the six target genes justified as follow: Serogroups A and E in duplex, Serogroups B and D in duplex, Serogroup F, *Kmt*1, *tox*A, *hgb*A, *sod*C and *nan*H genes in uniplex.

Table 2. Primers and probes used for Taqman Real-Time PCR Assays.

Target gene	Sequence (5'-3')	References
P. multocida **(*Kmt*1)**	ATAAGAAACGTAACTCAACATGGAAATA GAGTGGGCTTGTCGGTAGTCTT (FAM) AAACCGGCAAATAACAATAAGCTGA (BHQ1)	Sunaga *et al*. (2020)
P. multocida **serogroup A**	TTCGTTAAAAATGACAGCTATGC ATAATCGTCAGAAGCTCATGCG (FAM) TTTCTCAGCATTAACACATGATTGGAT (BHQ1)	Shikov *et al*. (2015)
P. multocida **serogroup B**	GCGTGTATAACCTACATCTTCCCA CGTCCATCAACACCTTTACTGC (R6G) TAGGCACAGAATATTCAAAACCCCGT (BHQ1)	Shikov *et al*. (2015)
P. multocida **serogroup D**	ATCGCATCCAGAATAGCAAACTC TCCGATGCTTTGGTTGTGC (Cy5) CGATTAAACTCAAATCTAGGGACATACTT (BHQ2)	Shikov *et al*. (2015)
P. multocida **serogroup E**	TGGGCACATGCTCGCTTA CTGCTTGATTTTGTCTTTCTCCTAA (ROX) ATGTGGCAAAGCGATCAATTCAGA (BHQ2)	Shikov *et al*. (2015)
P. multocida **serogroup F**	CGGAGAACGCAGAAATCAGAA CAACAACGACTTCAAATGGGTAG (R6G) CTTGCTCCATTGCCAGATCATGTT (BHQ1)	Shikov *et al*. (2015)
P. multocida **toxin (*tox*A)**	GAAATGGCTGGAAAAACCAGTG GAAAAGGCGCTGAAATTACTGTATC (FAM) CGGCTGATTTAATACGCTTTGCCTTGC (BHQ1)	Sunaga *et al*. (2020)

Pathological examination:

Tissue specimens from trachea and lungs were collected from 50 freshly dead and sacrificed rabbits then fixed in 10% buffered neutral formalin. Paraffin sections 5micron thick were prepared and stained with hematoxylin and eosin stain (Survarna *et al*., 2013) and examined microscopically.

RESULTS

Clinical and post mortem examination:

In order to identify the bacteria involved in rabbit-respiratory diseases (snuffles), rabbits showing generalized respiratory disorders were chosen for bacteriological and microscopic examination. Most of the examined rabbits showed coughing and sneezing with serous to mucoid or purulent nasal discharge. Snuffling sound has also been observed with the affected rabbits in addition to dyspnea, depression, anorexia and high body temperature.

Postmortem examinations of sacrificed or freshly dead rabbits revealed mild to severe congestion in trachea and lung with yellowish to grey exudates in some cases. Other cases showed spongy and edematous lung while bleeding from severed surfaces was observed in some cases.

Bacterial culturing:

Sixty-eight out of the 80 examined samples including nasal swabs, tracheal and lung tissues, were positively tested for *P. multocida*, *S. aureus* and *Mycoplasma* sp. The bacteriological analysis was primarily based on the characteristic colony morphology of the isolates on specific media including the typical "fried-egg" appearance of *Mycoplasma* on PPLO media (Fig. S1) followed by biochemical identifications; glucose fermentation positive, arginine and film &spot negative. The incidence rate for the three tested pathogens was approximately 85%, with recovery rates of 37.5%, 30% and 11.25% for *P. multocida*, *S. aureus* and *Mycoplasma* sp., respectively. The results illustrated in **table (3)** revealed that infection process occurred either in single or mixed form and the most common bacterial combination of mixed infection was *Mycoplasma* sp. with *S. aureus* or *Mycoplasma* sp. with *P. multocida*.

Table 3. Incidence of *P. multocida*, *S. aureus* and *Mycoplasma* sp. recovered from diseased and dead rabbits

Bacterial isolates	Examined sample, n = 80						Total
	Nasal swab, n = 30		Trachea, n = 25		Lung, n =25		
	single	Mixed	single	mixed	single	mixed	
P. multocida	8		8		10		26
***P. multocida + Mycoplasma* sp.**		0		1		3	4
TOTAL *P. multocida*	30 (37. 5%)						
S. aureus	7		4		11		22
***S. aureus +Mycoplasma* sp.**		0		1		1	2
TOTAL *S. aureus*	24 (30%)						
***Mycoplasma* sp.**	0		1		2		3
Mycoplasma* sp. + *P. multocida		0		1		3	4
Mycoplasma* sp. + *S. aureus		0		1		1	2
TOTAL *Mycoplasma* sp.	9 (11.25%)						

Molecular identification:

Molecular identification of *Mycoplasma* isolates. The bacteriologically positive Mycoplasma isolates were molecularly confirmed using a standard PCR approach targeting the 16SrRNA gene to reveal a unique DNA fragment at 240bp (Fig. 1a). Furthermore, a purified Mycoplasma PCR product of the 16SrRNA gene was subjected to sequencing in both directions. The original sequence was edited to remove the indeterminate sequences that are common at the start of a sequence reaction. The sequencing results verified that the isolate is Mycoplasma sp., and the sequences were submitted to the Genbank database under the accession number "MW132895". Phylogenetic analysis of *16SrRNA* sequence showed a close homology (95-100%) with field and reference strains of *Mycoplasma* species on comparison and designated the rabbit isolate as "RT11-*Mycoplasma* sp." as shown in the **(Figure 1b) & Table (4)**.

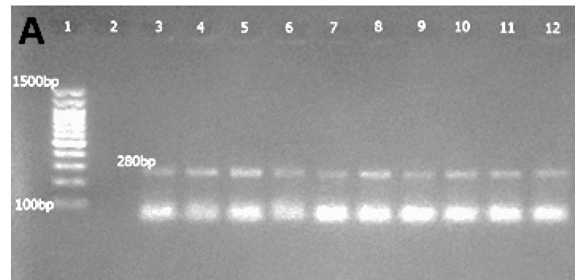


Fig. 1.A. Electrophoretic pattern of *16SrRNA* gene of *Mycoplasma* sp. Lane (1): 100 base pair DNA ladder. Lane (2): control negative. Lane (3): control positive. Lanes (4-12): positive amplifications for target gene at 280bp.

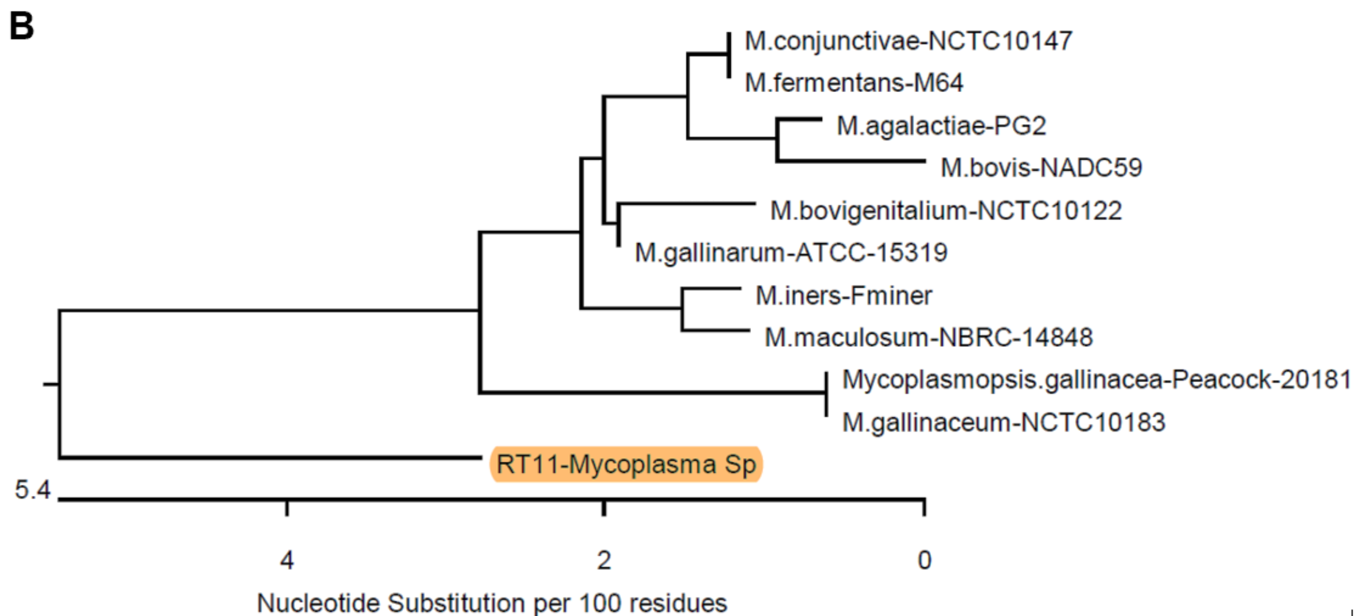


Fig. 1.B. Phylogenetic tree of nucleotide of *16SrRNA* gene of RT11-Mycoplasma sp. compared with reference and field strains

Table 4. Identity percent of nucleotide sequencing of *16SrRNA* gene of RT11-Mycoplasma sp. compared with reference and field strains

Percent Identity (upper triangle) / Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11		
1		93.3	93.3	93.3	92.9	92.9	92.9	92.9	94.0	93.6	93.6	1	RT11-Mycoplasma Sp
2	7.1		100.0	99.2	96.4	98.0	98.4	96.4	99.2	98.0	98.0	2	M.conjunctivae-NCTC10147
3	7.1	0.0		99.2	96.4	98.0	98.4	96.4	99.2	98.0	98.0	3	M.fermentans-M64
4	7.1	0.8	0.8		95.7	98.8	97.6	95.7	98.4	97.2	97.2	4	M.agalactiae-PG2
5	7.5	3.7	3.7	4.5		95.3	96.4	100.0	97.2	96.0	96.0	5	Mycoplasmopsis.gallinacea-Peacock-20181
6	7.5	2.0	2.0	1.2	4.9		97.2	95.3	98.0	97.2	96.8	6	M.bovis-NADC59
7	7.5	1.6	1.6	2.4	3.6	2.8		96.4	99.2	98.0	98.0	7	M.bovigenitalium-NCTC10122
8	7.5	3.7	3.7	4.5	0.0	4.9	3.6		97.2	96.0	96.0	8	M.gallinaceum-NCTC10183
9	6.3	0.8	0.8	1.6	2.9	2.0	0.8	2.9		98.8	98.8	9	M.gallinarum-ATCC-15319
10	6.7	2.0	2.0	2.9	4.1	2.9	2.0	4.1	1.2		99.2	10	M.iners-Fminer
11	6.7	2.0	2.0	2.9	4.1	3.3	2.0	4.1	1.2	0.8		11	M.maculosum-NBRC-14848
	1	2	3	4	5	6	7	8	9	10	11		

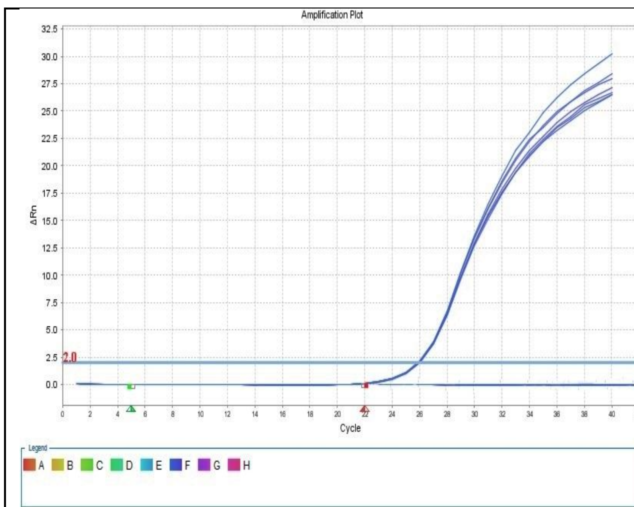
Molecular identifications of *Pasteurella* and *Staphylococcus* isolates:

RT-PCR was used to test the culture positive Pasteurella isolates using *Pasteurella multocida* specific primers generated from the kmt1 gene. As shown in Fig. (2.a), all isolates were verified to be *P. multocida*. Multiplex PCR was subsequently utilised to determine the capsular type of *P. multocida* isolates, as shown in Fig. (2b) (Fig. 2b). Serogroup A is assigned to all isolates. Concerning Staphylococcus isolates, the positive culture result for S. aureus, including biochemical characterisation, agreed 100 percent with the RT-PCR result, which found the 16SrRNA gene in all isolates. (**Fig. 2c**).

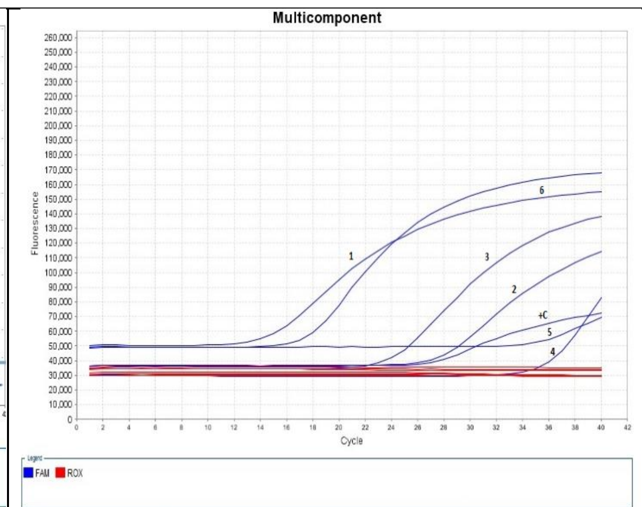
Furthermore, RT-PCR is used to detect the virulence associated genes in *P. multocida* including *sodC*, *hgbA* and *toxA* (**Fig. 2d-f**). The distribution of the tested virulence genes is presented in **Table (5)**, where, 100% of tested isolates were found to be positive for *sod*C gene, however, *hgb*A and *tox*A genes were detected in 83.3% and 33.3% of the tested isolates respectively, but none of the isolates tested positive for *nan*H gene. RT-PCR is also applied to identify *spa* and *ica* genes in *S. aureus* isolates indicating the presence of *spa* gene in 5 out of 7 tested isolates, while *ica* gene was found in all the 7 tested isolates (**Fig. 2g, h**) (**Table 5**).

Table 5. Detection of virulence genes in *P. multocida* and *S. aureus*

P. multocida		***S. aureus***	
No. of tested isolates (6)	**Virulence genes**	**No. of tested isolates (7)**	**Virulence genes**
3	*sod*C + *hgb*A	5	*spa* + *ica*
2	*sod*C + *hgb*A + *tox*A	2	*ica*
1	*sod*C		



a



b

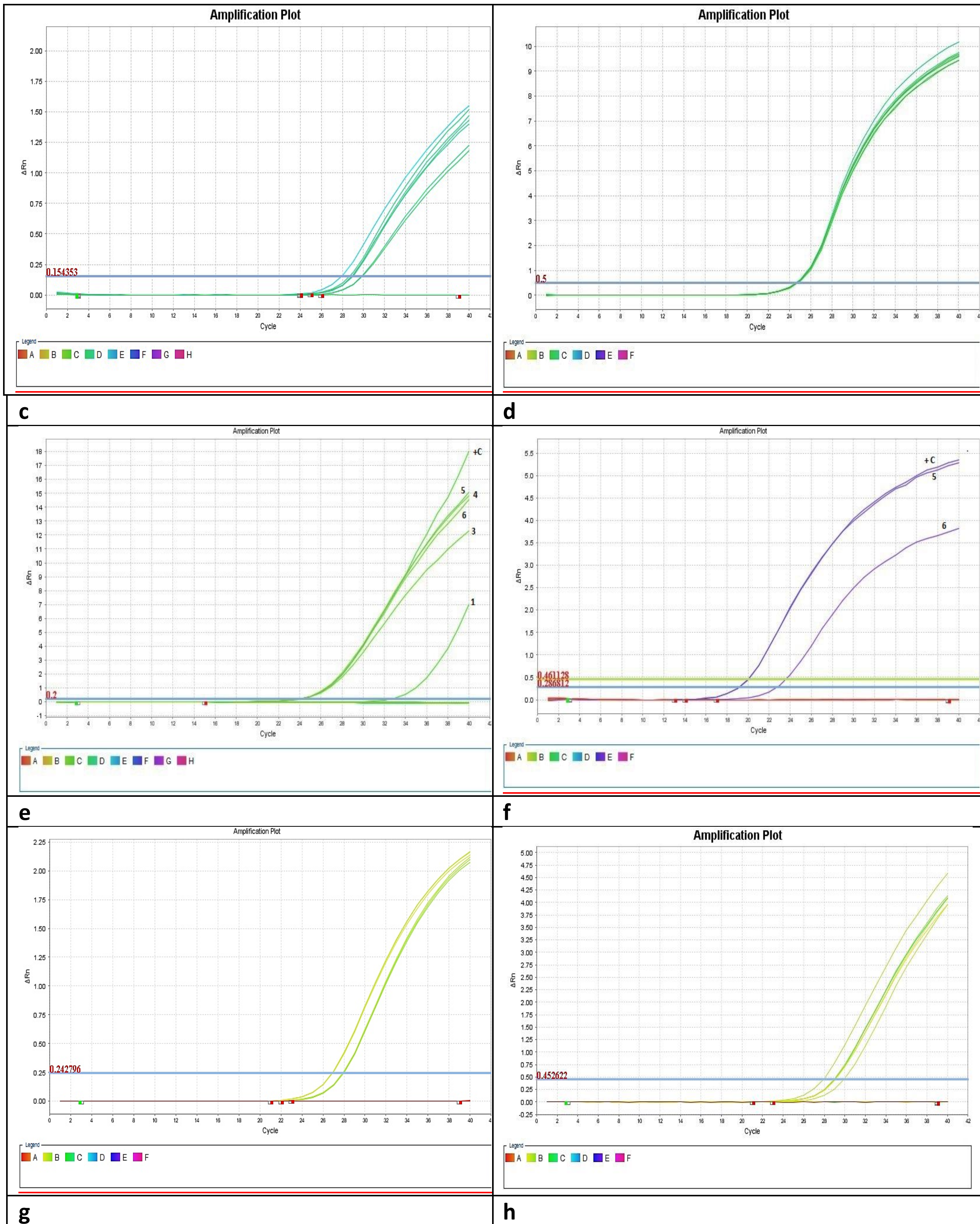


Fig. 2. PCR amplification plots identify the amplification plots that cross the threshold limits between 5 and 35 cycles as PCR-positive, where upper curves represent control postive: **P. multocida* (*P. multocida* subsp. *multocida* strain HN06) or *S. aureus* control positive (*S. aureus* 04-02981), lower linear curves represent control negative: PCR mixture without DNA. **a):** Taqman RT- PCR amplification plot for Kmt1 gene among 6 tested *Pasteurella* isolates. **b)** Multiplex amplification plot obtained with a detection system based on the use of two fluorophores with 6 tested *P. multocida* isolates: FAM (blue line) for the detection of serogroup A, VIC (red line) for the detection of serogroup B. **c):** SYBR Green RT-PCR amplification plot for positive detection of 16 sRNA among 7 tested *Staphylococci* isolates. **d):** SYBR Green RT- PCR amplification plot for detecting SodC gene in 6 tested *P. multocida* isolates. **e):** SYBR Green RT-PCR amplification plot for detecting *hgb*A gene in *P. multocida.* **f):** Taqman RT- PCR amplification plot for detecting ToxA gene in 6 tested *P. multocida* isolates **g):** SYBR Green RT-PCR amplification plot for detecting spa gene in 7 tested *S. aureus* isolates. **h):** SYBR Green RT-PCR amplification plot detecting ica gene in 7 tested *S. aureus* isolates.

Microscopic findings:

As illustrated in Table (6), the incidence and prevalence of microscopic lesions varied according to the involved etiological agent. Where, microscopic examination of trachea from rabbit with mycoplasmosis showed partial deciliation with submucosal edema (Fig. 3a) but in case of *P. multocida*, trachea showed submucosal infiltration with leukocytic cells (Fig. 3b) while, there was metaplasia of tracheal epithelial cells into goblet cells with destruction of submucosal tissue in case of *S. aureus* infection (Fig. 3c). Congestion was a common lesion reported in most infected rabbits with the aforementioned isolated microorganisms. Lung lesions also differ according to the causative agent. Lung lesions associated with mycoplasmosis include endotheliosis a nd vacuolation of tunica media in pulmonary blood vessels(Fig. 3d) and severe perivascular fibrosis with congestion (Fig. 4a), as well as, focal aggregation of lymphocytic cells (Fig. 4b).

In case of *S. aureus* infection, lung showed mild focal lymphocytic cells infiltration (Fig. 4c). While, there was diffuse emphysema and focal hemorrhage associated with most cases infected with pasteurellosis (Fig. 4d). The demonstrated lesions declared the microscopic lesions concerned with each single pathogen while those concerned with mixed ones were detailed in the lesion score (table S1). The mixed infections exaggerated the microscopic lesions showed in the single ones.

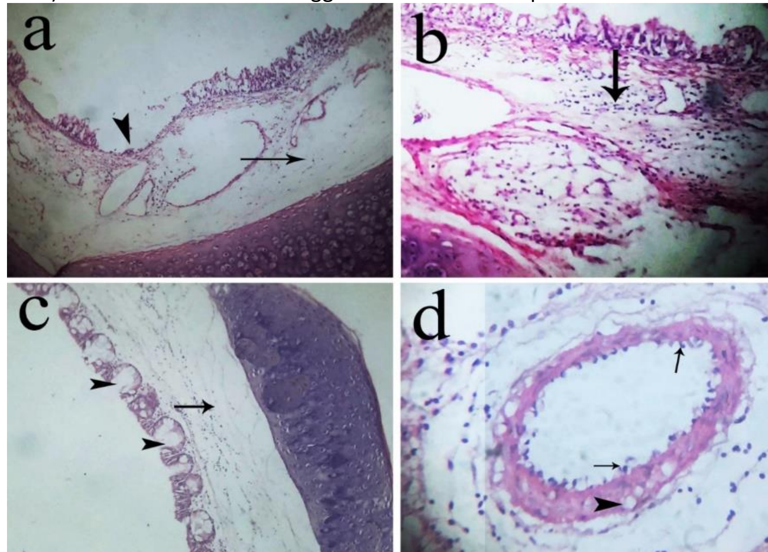


Fig. 3. Photomicrograph of trachea and lung of rabbits showing respiratory disorder (H&E x200). a): Trachea of infected rabbit with *Mycoplasma* sp. showing partial deciliation (head arrow) with partial destruction of submucosa (arrow). **b):** Trachea of infected rabbit with *P. multocida* showing submucosal infiltration with mononuclear leukocytic cells (arrow)**. c):** Trachea of infected rabbit with *S. aureus* showing metaplasia of epithelial cells into goblet cells (head arrows) with destruction of submucosal tissue (arrow). **d):** Lung of infected rabbit with *Mycoplasma* sp. showing endotheliosis (arrows) with vacuolation of tunica media (head arrow)**.**

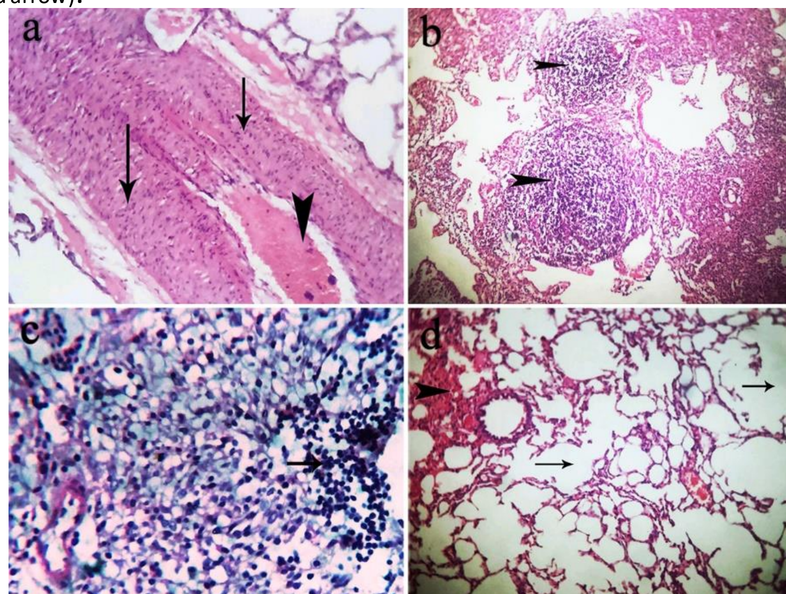


Fig. 4. Photomicrograph of trachea and lung of rabbits with respiratory disorders (H&E x400). a) Lung of infected rabbit with *Mycoplasma* sp. showing perivascular fibrosis (arrows) with congestion (head arrow). **b):** Lung of infected rabbit with *Mycoplasma* sp. showing focal aggregation of lymphocytic cells (head arrow) **(H&E x200)**. **c):** Lung of infected rabbit with *S. aureus* showing mild focal lymphocytic cells infiltration (arrow). **d)** Lung of infected rabbit with *P. multocida* showing diffuse emphysema (arrows) with focal hemorrhagic areas (head arrow).

DISCUSSION

Infections of the lungs continue to cause significant economic losses in domestic rabbits. The bacterial pathogens that are frequently isolated from affected rabbits are identified in relation to prevalence and pathogenicity in order to understand the pathogenicity of the bacteria involved in rabbit-respiratory disorders and identify the common bacterial combinations of concurrent infections and their contributions in the disease mechanisms for further control measures.

To begin, three bacterial pathogens, Mycoplasma, *P. multocida, and S. aureus*, were discovered to be implicated in rabbit respiratory illness based on bacteriological and molecular characterisation (snuffles). Mycoplasma pulmonis was shown to have a greater prevalence (43 percent) when co-infected with other pathogens such as *Bordetella bronchiseptica, P. multocidia,* and *Staphylococcus,* according to Villa *et al*. (2001). In rabbits with respiratory illness, these infections frequently present alone or in combination.

Regarding *P. multocida*, 37.5% incidence rate was reported in accordance with Ibtesam *et al*. (2013) who recorded the same incidence rate of *P. multocida* from nasal swabs and lung tissues. On the other hand, Wang *et al*. (2019) obtained an isolation rate of *P. multocida* from lungs of dead rabbits ranged between 21.2 and 53.1%, suggesting that *P. multocida* might be a major pathogen causing high mortalities in rabbit farms. The incidence rate of *S. aureus* (30%) in our study was found in line with what described by Eid and Samir, (2018), however, a lower incidence of *S. aureus* is reported by Abd El-Tawab *et al.* (2014). In the contrary, Wang *et al*. (2019) demonstrated high incidence of *S. aureus* that reached 52.9% and led to high mortality rates and significant economic losses in nine rabbit farms.

Next, molecular techniques including PCR and RT-PCR are used for further verification of the culture results with high sensitivity and specificity (Dutta *et al*., 2005). Thus, in order to confirm the positive *Mycoplasma* culture result, a *16S rRNA*-based mycoplasma group-specific PCR is used to identify the *Mycoplasma* isolates (Van Kuppeveld *et al*., 1994). Further, the PCR-product (*16SrRNA* gene) of one isolate was subjected to partial sequencing and phylogenetically characterized as *Mycoplasma* sp. The partial sequence of *16SrRNA* gene generated from the rabbit isolate showed 95-100% similarity with sequences of many strains from different *Mycoplasma* species including *M. conjunctiviae*, *M. agalactia*, *M. fermentans*, *M. gallinarum* in comparison. Such result indicates the implication of *Mycoplasma* sp. in respiratory diseases in domestic rabbit. Partial sequencing based on 280bp of 16SrRNA genes was found very limited in identifying *Mycoplasma* species and further sequencing of the whole genome might help in specific identification of the involved *Mycoplasma* species.

Furthermore, RT-PCR analysis verified the detection of *P. multocida* and *S. aureus* in rabbits. All *P. multocida* isolates were also typed as serotype A using multiplex RT-PCR, in agreement with recent study that found *P. multocida* of capsular type A is the predominant type associated with rabbits-respiratory disease (Massacci *et al*., 2018).

The pathogenicity and host specificity of different microorganisms are usually attributed to the presence of certain virulence genes that describe their epidemiology and pathogenic mechanisms. In the current study, we screened the existence of four virulence genes associated with pathogenicity of *P. mulocida* including; hemoglobin-binding proteins (*hgb*A), superoxide dismutase (*sod*C), neuraminidase (*nan*H) and dermonecrotic toxin (*tox*A). Our results detected *tox*A in 33.3% of *P. multocida* isolates, while *hgb*A and *sod*C were found in 83.3% and 100% of the examined isolates, respectively. But *nan*H wasn't detected in any isolates. In comparison, all isolates reported by Prajapati *et al*. (2020) carried *sod*C gene, and 37% of isolates express *hgb*A gene, but, *nan*H was found in 90% of the isolates.

The virulence and pathogenicity of *S. aureus* has been linked to expression of *S. aureus* protein A *(spa*) and the intercellular adhesion (*ica*) genes (Paniagua *et al*., 2014). As described in an earlier study, 71.4% of the current reported isolates carried *ica* and *spa* gene, with an increased incidence of *spa* gene (93.8%) as a major virulent protein contributing to severity of respiratory infections (Bhati *et al*., 2016). Similarly, Abd El-Tawab *et al*. (2014) and Arciola *et al*. (2003) acknowledge the role of *ica* operon in synthesis of the capsular polysaccharide, which supports the cell-to-cell bacterial contacts by means of a multilayered biofilm. In contrast, *ica* gene has been shown to decrease in other studies (Viana *et al*., 2012).

In order to further our understanding of the disease pathogenicity in relation to the involved pathogens, gross and microscopic lesions are assessed, reporting variation in lesions according to the causative agent. However, common lesions of congestion and haemorrhage are reported in either single or mixed infection of the three detected bacteria. However, it is worth noting that mixed infections including Mycoplasma as a major partner resulted in exaggerated disease severity. It suggests that Mycoplasma contributes to downing the host defense mechanism, allowing *P. multocida* or *S. aureus* to establish and develop a severe form of infection in the respiratory tract of rabbits. Generally, gross lesions associated with mycoplasmosis in rabbits range from serous to supportive exudates in the nasal passages and airways in addition to a variable degree of pulmonary congestion. Similar observations were reported by Langan *et al*. (2000) in rabbits infected with *M. pulmonis*. The microscopic lesions currently observed with mycoplasmosis including deciliation of trachea, and edema of tracheal lamina propria, were consistent with the previous finding of Marien (2007). Endotheliosis, focal lymphocytic infiltration and leukocytic infiltration were also prominent lesions in lung of rabbits infected with *Mycoplasma* as a single or mixed infection. These observations were verified by George *et al*. (2000) who documented three distinct microscopic lesions induced by *M. pulmonis* in the airway of rodents, including neutrophil infiltration, epithelial hyperplasia, and lymphocytic hyperplasia in the lamina propria of the submucosa. We hypothesized that leukocytosis and lymphocyte infiltration may be attributed to the infection-related inflammatory condition. Our alternative hypothesis is in agreement with Pravina *et al.* (2010) who believed that infiltration of these cells into the infected tissues might result from apoptosis induced by bacterial toxins.

Grossly, tracheal lesions in case of *S. aureus* or *P. multocida*, were characterized by congestion with yellowish to greyish exudate, in consistent with what previously reported by Premalatha *et al.* (2009). Microscopically, lesions include congestion of tracheal and pulmonary blood vessels with leukocytic cells infiltration, as well as metaplasia of tracheal epithelial cells into goblet cells. Increased goblet cells in the respiratory epithelium either in single or mixed infections is a logical feature because goblet cells participate in the production of mucin as a host defense mechanism against infection especially in the respiratory tract (Scharfman *et al.,* 1996). The current finding also support those of Percy *et al.*, (1986) who noticed catarrhal trachitis in

rabbits infected with *S. aureus* and *P. multocida*. The major detected vascular lesions including pulmonary congestion and hemorrhages were in line with observation of Cynthia. (2005) Anitha and Mammen (2013).

CONCLUSION

Finally, the findings provided in this study demonstrated for the first time the importance of Mycoplasma sp. infection, either alone or in combination with other bacterial pathogens (*S. aureus* and *P. multocida*), in the development of severe respiratory illnesses or snuffles syndrome in rabbits. By using culture/PCR and RT-PCR techniques, the researchers were able to recover virulent strains of *S. aureus* and *P. multocida* isolates from rabbits with respiratory illnesses, suggesting the harmful impact of such bacterial infections on Egypt's rabbit industry.

Conflict of interest: No conflict of interest to declare.

The authors contribution: Authors contributed equally to the work, and the manuscript was written by Hala R. Ali.

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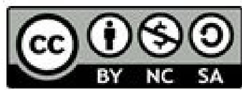

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التوصيف الجزيئي لمسببات الأمراض البكتيرية المسببة للشخير في الأرانب من حيث الانتشار والإمراضية

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الملخص العربى

تعتبر أمراض الجهاز التنفسي مشكلة رئيسية مرتبطة بخسائر اقتصادية جسيمة في صناعة الأرانب . إن سبب المرض في الأرانب هو عدوى متعددة البكتيريا لأنها تنتج عن إصابة مشتركة بأكثر من مسبب من مسببات الأمراض البكتيرية . لتحديد مسببات الأمراض البكتيرية المتضمنة في التهابات الجهاز التنفسي خاصة العدوى المختلطة من حيث الانتشار والإمراضية، فإن 80 عينة من الأرانب المصابة باضطرابات تنفسية نموذجية قد خضعت للفحص البكتيري متبوعًا بالتشخيص الجزيئي من خلال qRT-PCR أو PCR ثم التتابع النيوكليوتيدى . ومع ذلك، فإن تسلسل 16SrRNAجين لعزل الميكوبلازما لم يستطيع تحديد أنواع الميكوبلازما المعزولة من الأرانب . ولذلك فقد تقتضى الحاجة إلى مزيد من العمل . وقد حددت النتائج وجود عترات الميكوبلازما كشريك رئيسي في العدوى المختلطة مع الباستيريلا مالتوسيدا والاستافيلوكوكس أوريس، مما ساهم في تطوير شكل حدية أمراض الجهاز التنفسي فى الأرانب . كما تم اجراء الفحص الباثولوجي والميكروسكوبى لإثبات الآفات المصحوبة بكل عامل بكتيري . أشارت نتائجنا أيضًا إلى ارتفاع معدل انتشار السلالات الأكثر ضراوة من الباستيريلا مالتوسيدا والاستافيلوكوكس أوريس مما يشير إلى التأثير السلبي لهذه المسببات البكتيرية على صناعة الأرانب .

الكلمات المفتاحية: الأرانب ، الميكوبلازما ، المكورات العنقودية الذهبية ، التشريح المرضي ، تفاعل البلمرة المتسلسل RT- PCR