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BACTERIOLOGICAL STUDIES ON SPORADIC SUDDEN DEATH CASES IN RABBIT FARMS USING MULTIPLEX PCR

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ABSTRACT: Rabbits rearing is worldwide rapid well developed industry especially in developing countries as it resemble a good alternative cheap, and high source of animal protein need less rearing areas with low costs in comparison to red meat sources. Many challenges face this industry include viral, bacterial, parasitic, nutritional deficiencies and mis-management diseases. Although the weaned rabbits as well as rabbits dams were considered as the big income of rabbits producers, they attacked by certain undiagnosed pathogenic agents that cause sporadic deaths allover rabbits farms resulting in economic losses. So this work was aimed to focus on bacterial causes of the sporadic rabbits mortalities using Multiplex PCR which detect multiple bacteria at the same time in many samples. Lungs and/or livers samples were collected from freshly dead rabbits of 20 rabbit farms under complete aseptic conditions and preserved in refrigerator till sent rapidly to lab. for Multiplex PCR application against 8 available primers of *M. haemolytica*, *Mycoplasma* spp, *Klebsiella* spp, *E. coli* spp, *P. multocida*, *Y. enterocolitica*, *Staphylococcus* spp, and *L. momnocytogens*. Eighteen rabbits farms affected with *E. coli* spp, followed by *Staphylococcus* spp (11), *Klebsiella* spp (9), *Y. enterocolitica* (7), and *Mycoplasma* spp (2). *L. momnocytogens*, *P. multocida*, and *M. haemolytica* were not detected in rabbits farms. One farm had no infection, five farms had a single infection, and fourteen farms had mixed infection with *E. coli*. This study concluded that sporadic sudden death cases in rabbits farms were recorded mostly in weaned, and juvenile rabbits more than adult ones, *E. coli* is the most primary causative agent with those cases, *Mycoplasma* was a new interest should be considered in such cases where it was recovered from adult rabbits dams, and finally *Klebsiella*, and *Y. enterocolitica* should be taken in consideration on sporadic mortality particularly in weaned and juvenile rabbits.

Keywords: Bacteria, Multiplex PCR, Rabbits, sudden death

INTRODUCTION

Family Leporidae, and order Lagomorpha include hares and Rabbits which are used in human feeding, fur industry, and biomedical research (Catty,1988).

The red meat animal protein have a shortage all over the world, so the Rabbit meat is considered a substitutive source which can solve this problem (Dalle and Szendro, 2011).

Although, Domestic and wild rabbits have an economic, ecological, and public health importance, the information on rabbits medicine and pathology as well as the published research papers of diagnostic cases in rabbits are rare (Espinosa et al., 2020).

The onset of diseases couldn't be avoided, despite the progression in genetics, feeding, management, and increased productivity. The disease or low productivity resulting in the high percent of culled animals from farms (Sánchez et al., 2004 and EFSA, 2005).

rabbit breeding suffered from significant economic losses caused by Digestive infections (Saravia et al., 2017) including parasitic, bacterial, and viral (Langan et al., 2000; Lennox and Kelleher, 2009).

Harkness et al., 2010 reported the death syndrome in rabbit Suddenly without preceding signs might have many etiologies including microbial infections, stresses, gastro-intestino pathies, and intoxications. Also, Ali Shalizar-Jalali et al., 2019 recorded sudden death without any clinical signs in White New Zealand female rabbit caused by *Eimeria stiedae* oocytes affected liver tissue with severe bile ducts hyperplasia and dilatation.

Young rabbits were negatively affected with Bacterial infections resulting in reduced body weight and high mortality (Zahraei et al., 2010).

Although, the gastrointestinal tract of warm-blooded animals have the common commensal bacteria, *Escherichia coli* (*E. coli*), certain strains become virulent expressing diarrheal and extra-intestinal diseases in an immune-suppressed host (Croxen et al., 2013).

Both *E. coli*, and *Salmonella enterica* were considered a potential risk in rabbit flocks particularly the newborn New Zealand rabbits with diarrhea, and hemorrhagic colitis (Hamed et al., 2013; Suelam and Reda, 2015).

young rabbits orally inoculated with *Yersenia enterocolitica* revealed clear diarrhea and systemic invasion like that of child (Heesemann et al., 1988 and O'Loughlin et al., 1988).

Swennes et al., (2012) Identified many bacteria like *Enterobacter*, *Pseudomonas*, *Streptococcus*, *Klebsiella*, *Escherichia*, *Staphylococcus*, *Bacillus*, and *Proteus* from Fecal samples of laboratory rabbits.

Profuse mucoid or bloody watery diarrhea in rabbits mainly caused by *Entero-pathogenic E. coli* (*EPEC*) and followed by lethargy, dehydration, and anorexia (Peeters et al., 1984 and Heczko et al., 2000).

EPEC was strictly identified in the gut of rabbits inducing inflammatory lesions and considered as the only known class of *E. coli* (Licois, 2004).

Not only, serious problems attack rabbits farming in Egypt but also the rabbits diseases that caused severe economic losses like viral and bacterial hemorrhagic septicemia have the great attention (Saif-Edin et al., 1994). So, this work interested to investigate the sudden sporadic deaths in rabbits referring to bacterial causes using multiplex PCR and used as an overview in control strategy of rabbit flocks to avoid the further economic losses.

MATERIALS AND METHODS

Samples collection and processing

Under complete hygienic conditions, samples from Lungs and/or livers of fresh, suddenly dead rabbits were collected from different ages and species of twenty rabbits farms at Alexandria governorate during the period from

August 2021 to February 2023. They exhibited sudden death without evidence to any apparent clinical signs except diarrhea at onset of death (table 1). These samples were preserved in refrigerator till sent rapidly to Reference laboratory for Veterinary Quality Control on Poultry Production, Agriculture Research Center, Egypt. for Multiplex PCR application against 8 available primers of *E. coli*, *Y. enterocolitica*, *Klebsiella*, *Staphylococcus*, *Mycoplasma*, *Listeria. momnocytogens*, *Pasteurella. multocida*, and *Mannheimia. haemolytica*.

DNA extraction

Qiagen, Germany, DNA Mini kit used to extract DNA as the following, mix 200 µl of the sample suspension with 10 µl of proteinase K and 200 µl of lysis buffer. All contents incubated at 56°C for 10 min. then add 200 µl of 100% ethanol and wash the sample followed by centrifugation and elution of nucleic acid with 100 µl of elution buffer.

Oligonucleotide Primers

Primers obtained from Metabion, Germany (table 2).

PCR amplification

Using the Applied Bio system 2720 thermal cycler, 25 µl of EmeraldAmp Max PCR Master Mix plus 1 µl from each primer (20 pmol concentration), 11 µl of water, and 6 µl of DNA template.

PCR Products Analysis

40 µl of the PCR products and Gelpilot 100 bp plus ladder were loaded in 1.5% agarose electrophoresis gel using 1x TBE buffer at room temperature (5V/cm). The gel documentation system (Alpha Innotech, Biometra) was photographed the gel and the computer software analyzed the data.

RESULTS

Multiplex PCR electrophoretic image

Samples taken from sudden dead rabbits exhibited negative results with primers of *M. hemolytica*, *P. multocida*, and *L. monocytogenes* at 325, 460, and 1200 bp, respectively and positive results with *Klebsiella*, *E. coli*, *Y. enterocolitica*, *Staphylococcus*, and *Mycoplasma* at 441, 720, 330, 791, and 1013 bp, respectively (Fig. 1).

Single and mixed infection

PCR results showed there was no bacterial infection in one out of twenty rabbit flocks, presence of single infection in five flocks (*Staphylococcus* in one, and *E. coli* in four flocks). While the mixed infection recorded in fourteen rabbit flocks, six dual infection, three of them were affected with *E. coli*, and *Staphylococcus*, one flocks was affected with *E. coli*, and *Mycoplasma*, one flocks was affected with *E. coli*, and *Y. enterocolitica*, and one flocks was affected with *E. coli*, and *Klebsiella*. Triple infection recorded in three flocks, two of them were affected with *E. coli*, *Staphylococcus*, and *Klebsiella* spp, another one was affected with *E. coli* spp, *Y. enterocolitica*, and *Klebsiella* spp. Tetra infection recorded in four flocks affected with *E. coli*, *Y. enterocolitica*, *Klebsiella*, and *Staphylococcus.* Finally, penta infection recorded in one flock affected with *E. coli*, *Y. enterocolitica*, *Klebsiella*, *Staphylococcus*, and *Mycoplasma* (Table 3 & 4).

Incidence of different bacteria in sudden dead rabbits

Ninty percent (90%) of an investigated rabbit flocks were affected with *E. coli*, 55% with *Staphylococcus* spp, 45% with *Klebsiella* spp, 35% with *Y. enterocolitica*, 10% with *Mycoplasma* spp, and finally 5% without an infection (Table 5).

DISCUSSION

The sporadic sudden mortality in rabbit farms all over the different ages was a major problem threaten this industry and may be caused by accidental, managemental, nutritional, and/or several pathogenic agents like viruses, bacteria and parasites. Virus characterized by high mortality, rapid onset, short course, and presence of clinical signs but these cases imitate the other causes where they occur with random, sudden, no apparent clinical signs and slow economic losses all over the all ages at different rabbit farms. So in this work the bacterial causes will be highlighted as a causative agents of the sporadic mortality in rabbit farms using Multiplex PCR as a rapid diagnostic technique detected multiple bacteria at the same time in the suddenly dead rabbits.

The results revealed 5% of affected rabbit flocks with no infection, 25% had a single infection, and finally mixed infection recorded in 70% of rabbit flocks as follow 30% dual infection, 15% Triple infection, 20% Tetra infection, and 5% penta infection. Also, *E. coli* recorded with the higher incidence followed by *Staphylococcus* spp, *Klebsiella* spp, *Y. enterocolitica*, and finally *Mycoplasma* spp. These results were nearly similar to Ali Shalizar-Jalali et al., (2019) stated that rabbits' sudden death caused by several etiologies including temperature changes, fear, dental problems, toxicity, parasitic infestations, and gastro-intestinal disorders. Espinosa et al., (2020) was investigated Between 2000 and 2018 rabbits and hares (n=325) of northern Spain and identified viral infections (n = 31; 11.61%), bacterial diseases (n = 56; 20.97%), parasitic conditions (n = 65; 24.34%), nutritional and metabolic disorders (n = 48; 17.97%), toxicoses (n = 11; 4.11%), congenital diseases (n = 4; 1.49%), neoplasms (n = 12; 4.49%), trauma-related injuries (n = 9; 3.37%) and miscellaneous causes (n = 31; 11.61%). Virginia et al., (2017) investigated 54 intestinal contents of rabbits affected with diarrhea in Mexico State, southeastern part and they found that the most prominent agent was *Eimeria spp.* (77.5%), then *Aeromonas spp.* (15.5%), and *Enteropathogenic E. coli* (8.6%). *Rotavirus*, *Enterococcus spp.*, *Klebsiella spp.*, Salmonella spp., *Mannheimia spp.*, *Streptococcus spp.*, and *Staphylococcus aureus* were also detected. Arafa et al., (2000) isolated *E. coli* at percentage of 53.7%, also, Saif-Eldin et al., (1994) recorded *E. coli* in 54% of diarrheic rabbits, while Swennes et al., (2013) identified it with 61% and Ebied, (2012) mentioned 64% of freshly dead rabbits affected with *E. coli*. Sakr et al., (2019) diagnosed *E. coli* at Alexandria governorate in freshly dead53.23%, diarrheic70.12%,, and apparently healthy rabbits 57.43%. Pai, et al., (1980) found *Y. enterocolitica* strains MCH-628 when inoculated into rabbits induced Diarrhea in 87% (20/23) while MCH-700 strain induced Diarrhea in 88% (21/24). Saad Eldin and Reda., (2016) identified diarrheagenic *E. coli* in suckling rabbits in Egypt. Eid et al., (2017) during January 2016-March 2017, examined 625 samples (livers, Intestines , spleens, kidneys and heart bloods) out of 125 diseased rabbits (1-2 months old) were suffering from colisepticaemia and found *E. coli* in 525 out of the 625 samples (84%). Livers, intestines, spleens, kidneys, and heart bloods, were positive for *E. coli*, 30.4%, 29%, 8.7%, 17.4%, and 14.5%, respectively at Port said Governorate

CONCLUSION

Sporadic sudden rabbit deaths were recorded in weaned, and juvenile more than adult rabbits, and caused primarily by *E. coli* and/or Staphylococcus as they recorded in a single infection. The new researches should be interested with *Klebsiella* and *Y. enterocolitica* particularly in juvenile and weaned rabbits. Mycoplasma recorded in adult rabbit dams which indicated a new interest should be considered in such sporadic sudden deaths. Further studies on these diagnosed M.Os as antibiogram, and virulence factors should be continued.

Table (1): History of collected samples from sudden death in rabbits flocks in Alexandria governorate

Flock NO.	Area	Date	Breed/age stage	NO. of sudden dead rabbit/ total NO.	Post Mortem lesions
1	Borg El-Arab	22/5/2021	California/Weaned	1/50	Slight congested lung, and liver
2	El-Sahel Kilo23	13/5/2022	New Zealand /weaned	2/150	Slight congested lung, and liver
3	Borg El-Arab	2/11/2021	Gabaly/Weaned	1/100	Slight congested lung, and liver
4	Borg El-Arab	11/6/2021	New Zealand /Jouvenile	2/100	Lung caseation
5	El-Amria	13/8/2021	New Zealand /Adult	1/40	Severe congestion in lung and liver, intestine
6	El-Amria	17/7/2021	New Zealand /Jouvenile	1/150	Severe congestion in lung and liver, intestine with diarrhea at onset of death
7	Abees	2/9/2022	Gabaly/Adult	1/150	Severe congestion in lung and liver, intestine with diarrhea at onset of death
8	Abees	3/4/2022	New Zealand /Adult	1/50	No apparent lesions
9	El-Nobaria	15/6/2021	New Zealand /weaned	1/100	No apparent lesions
10	El-Nobaria	13/8/2022	New Zealand /weaned	1/80	No apparent lesions
11	El-Sahel Kilo23	11/9/2022	Shanchilla/weaned	1/100	No apparent lesions
12	El-Sahel Kilo23	1/11/2022	New Zealand /weaned	1/50	Slight congested lung, liver with diarrhea
13	El-Sahel Kilo23	19/2/2023	New Zealand /weaned	2/100	Slight congested lung, liver with diarrhea at onset of death
14	Abees	15/6/2022	New Zealand /Adult	1/30	No apparent lesions
15	El-Nobaria	16/10/2021	New Zealand /weaned	2/200	Slight congested lung, liver with diarrhea at onset of death
16	El-Amria	25/1/2022	New Zealand /Adult	1/40	No apparent lesions
17	Borg El-Arab	13/8/2021	New Zealand /Jouvenile	1/50	Slight congested lung
18	El-Amria	2/9/2022	Gabaly/Jouvenile	1/30	Congested intestine with diarrhea at onset of death
19	El-Amria	17/11/2021	Balgyki/Adult	1/50	No apparent lesions
20	El-Amria	13/6/2022	New Zealand /Jouvenile	1/80	Lung caseation

Table (2): Primers sequences, target genes, amplicon sizes and cycling conditions.

Multiplex	Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)	Reference
A	*L. monocytogenes 16S rRNA*	ggA CCg ggg CTA ATA CCg AAT gAT AA	1200	Primary denaturation at 94˚C 5 min., secondary denaturation at 94˚C 30 sec., Annealing at 55˚C 40 sec., Extension at 72˚C 1.2 min., and Final extension at 72˚C 12 min.	**Kumar et al., 2015**
		TTC ATg TAg gCg AgT TgC AgC CTA			
	Y. enterocolitica 16S rRNA	AAT ACC GCA TAA CGT CTT CG	330		**Wannet *et al.*, 2001**
		CTT CTT CTG CGA GTA ACG TC			
	Staphylococcus 16S rRNA	CCTATAAGACTGGGATAACTTCGGG	791		**Mason *et al.*, 2001**
		CTTTGAGTTTCAACCTTGCGGTCG			
	P. multocida Kmt1	ATCCGCTATTTACCCAGTGG	460		**Oie, 2012**
		GCTGTAAACGAACTCGCCAC			
B	*M. hemolytica ssa*	TTCACATCTTCATCCTC	325	Primary denaturation at 95˚C 5 min., secondary denaturation at 94˚C 30 sec., Annealing at 50˚C 40 sec., Extension at 72˚C 1 min., and Final extension at 72˚C 10 min.	**Hawari *et al.*, 2008**
		TTTTCATCCTCTTCGTC			
	E. coli phoA	CGATTCTGGAAATGGCAAAAG	720		**Hu *et al.*, 2011**
		CGTGATCAGCGGTGACTATGAC			
	Klebsiella gyrA	CGC GTA CTA TAC GCC ATG AAC GTA	441		**Brisse and Verhoef, 2001**
		ACC GTT GAT CAC TTC GGT CAG G			
	Mycoplasma 16S rRNA	GCTGGCTGTGTGCCTAATACA	1013		**Sayin *et al.*, 2016**
		TGCACCATCTGTCACTCTGTTAACCTC			

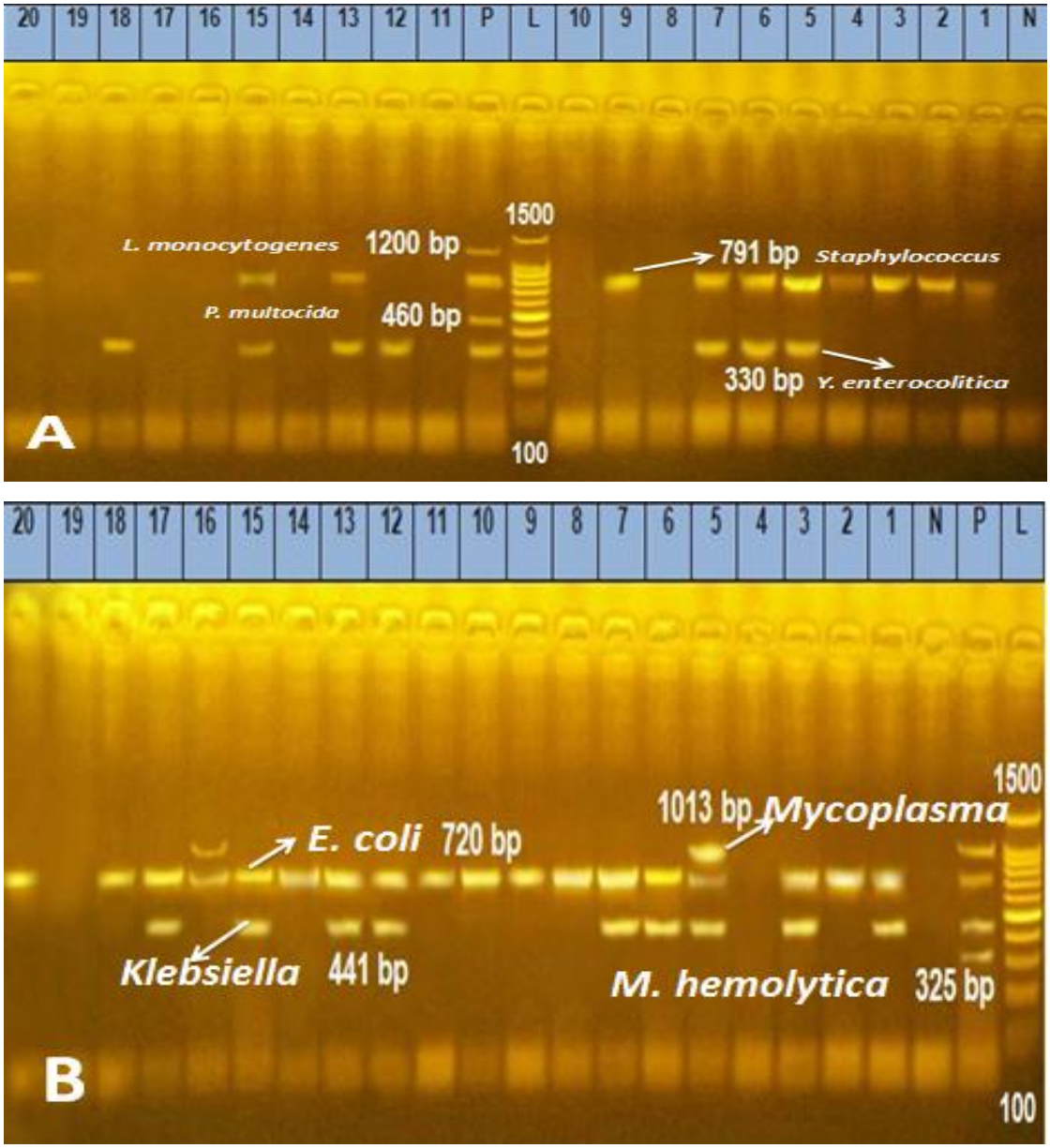

Figure (1): Lane P is positive control.. Lane N is negative control. Lane L is ladder. All samples were negative for *L. monocytogenes*, *P. multocida*, and *M. hemolytica* at 1200, 460, and 325 bp, respectively. Multiplex PCR A: Lane 1-7, 9, 13, 15, 20 showed samples were positive for *Staphylococcus* at 791 bp. Lane 5-7, 12, 13, 15, 18 were positive for *Y. enterocolitica* at 330 bp. B: Lane 5, 16 were positive for *Mycoplasma* at 1013 bp.. Lane 1-3, 5-18, 20 were positive for *E. coli* at 720 bp. Lane 1, 3, 5, 6, 7, 12, 13, 15, 17 were positive for *Klebsiella*

Table (3): Multiplex PCR results in affected rabbits farms with sporadic sudden death cases

Farm No.	Breed of examined rabbit	*E. col* spp	*Y. enterocolitica*	*Klebsiella* spp	*Staphylococcus* spp	*P. multocida*	*M. hemolytica*	*L. monocytogenes*	*Mycoplasma* spp
1	California/Weaned	+	-	+	+	-	-	-	-
2	New Zealand /weaned	+	-	-	+	-	-	-	-
3	Gabaly/Weaned	+	-	+	+	-	-	-	-
4	New Zealand /Jouvenile	-	-	-	+	-	-	-	-
5	New Zealand /Adult	+	+	+	+	-	-	-	+
6	New Zealand /Jouvenile	+	+	+	+	-	-	-	-
7	Gabaly/Adult	+	+	+	+	-	-	-	-
8	New Zealand /Adult	+	-	-	-	-	-	-	-
9	New Zealand /weaned	+	-	-	+	-	-	-	-
10	New Zealand /weaned	+	-	-	-	-	-	-	-
11	Shanchilla/weaned	+	-	-	-	-	-	-	-
12	New Zealand /weaned	+	+	+	-	-	-	-	-
13	New Zealand /weaned	+	+	+	+	-	-	-	-
14	New Zealand /Adult	+	-	-	-	-	-	-	-
15	New Zealand /weaned	+	+	+	+	-	-	-	-
16	New Zealand /Adult	+	-	-	-	-	-	-	+
17	New Zealand /Jouvenile	+	-	+	-	-	-	-	-
18	Gabaly/Jouvenile	+	+	-	-	-	-	-	-
19	Balgyki/Adult	-	-	-	-	-	-	-	-
20	New Zealand /Jouvenile	+	-	-	+	-	-	-	-
Total /20		18	7	9	11	-	-	-	2
Percentage %		90	35	45	55	-	-	-	10

+= Positive **- = Negative**

Table (4): Single and mixed infection in affected rabbit flocks with sporadic sudden death cases

Type of infection	Number of affected rabbit flocks	%	Identified bacteria
No infection	1	5%	_
Single	4	25%	*E. coli*
	1		*Staphylococcus*
Dual	3	30%	*E. coli*, and *Staphylococcus*
	1		*E. coli*, and *Mycoplasma*
	1		*E. coli*, and *Klebsiella*
	1		*E. coli*, and *Y. enterocolitica*
Triple	2	15%	*E. coli*, *Staphylococcus*, and *Klebsiella*
	1		*E. coli*, *Y. enterocolitica*, and *Klebsiella*
Tetra	4	20%	*E. coli*, *Y. enterocolitica*, *Klebsiella*, and *Staphylococcus*
Penta	1	5%	*E. coli*, *Y. enterocolitica*, *Klebsiella*, *Staphylococcus* and *Mycoplasma*
Total	20/20	100%	_

Table (5): Incidence of identified bacteria in affected rabbit flocks with sporadic sudden death cases

Identified bacteria	Single infection	Mixed infection	Total	percent
E. coli spp	4	14	18/20	90%
Y. enterocolitica	-	7	7/20	35%
Klebsiella spp	-	9	9/20	45%
Staphylococcus spp	1	10	11/20	55%
P. multocida	-	-	-	-
M. hemolytica	-	-	-	-
L. monocytogenes	-	-	-	-
Mycoplasma spp	-	2	2/20	10%

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الملخص العربى

دراسات بكتيرية على حالات الموت الفردي المفاجئ في مزارع الارانب باستخدام تفاعل البلمرة المتسلسل المتعدد

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تعتبر تربية الأرانب من الصناعات سريعة النمو والتطور وخاصة في الدول النامية حيث أنها تعد كبديل جيد ورخيص للحوم الحمراء وكمصدر عالي للبروتين الحيواني ولا تحتاج لمساحات كبيرة للتربية ولكن هناك الكثير من التحديات تواجه تلك الصناعة منها العدوي الفيروسية والبكتيرية والطفيلية ومشاكل النقص الغذائي وعدم الرعاية الجيدة. وعلي الرغم من أن الارانب المفطومة والأمهات تمثل المصدر الأكبر للدخل لدي منتجي الأرانب الا أن هناك مجموعة من العوامل الممرضة الغير مشخصة والمتسببة في حدوث الموت لتلك الأرانب بصورة فردية في كل مزارع الأرانب مما يؤدي الي حدوث خسائر اقتصادية لذلك جاء هذا العمل البحثي للتركيز علي المسببات البكتيرية لحالات الوفيات الفردية وتشخيص تلك المسببات بتفاعل البلمرة المتسلسل المتعدد والذي من خلاله يمكن فحص أكثر من عينة لأكثر من نوع بكتيريا في ذات الوقت. تم تجميع العينات من عشرون مزرعة أرانب متمثلة في الرئة والكبد من الأرانب حديثة النفوق وذلك باتباع وسائل التعقيم الكاملة والاجراءات الصحية وتم ارسال العينات الي المعمل المرجعي للرقابة علي الانتاج الداجني للفحص باستخدام جهاز البلمرة المتسلسل المتعدد في وجود البرايمرات المتاحة لعدد 8 بكتيريا وتشمل المنهيميا هيموليتيكا والميكوبلازما والكليبسيلا والايشريشيا كولاي والباستيريلا مالتوسيدا و اليرسينيا انتيروكوليتيكا والاستاف و والليستريا مونوسيتوجين وكانت النتائج كالتالي الاصابة بعدوي الميكروب القولوني ايشرشيا كولاي في 18 مزرعة يليها اصابة عدد 11 مزرعة بالاستاف و9 مزارع بالكليبسيلا و7 مزارع باليرسينيا انتيروكوليتيكا و2 مزرعة بالميكوبلازما. من اجمالي 20 مزرعة عدد 5 مزارع تمت اصابتها بعدوي فردية وعدد 14 مزرعة بعدوي مختلطة مع الايشريشيا كولاي ومزرعة واحدة بدون أي اصابات وخلصت هذه الدراسة الي أن حالات الموت الفردي المفاجئ في مزارع الأرانب كان الأكثر حدوثا في الأرانب المفطومة والشابة عنها في الأرانب البالغة كما أن بكتيريا الايشريشيا كولاي تعتبر المسبب الرئيسي لتلك الحالات. الميكوبلازما يجب الاهتمام بها حيث تم تشخيصها في الارانب البالغة وأخيرا بكتيريا الكليبسيلا واليرسينيا انتيروكوليتيكا لابد أن تؤخذ بعين الاعتبار في الحالات الفردية للموت المفاجئ وبصفة خاصة في الارانب المفطومة والشابة.

الكلمات الدالة: بكتيريا وتفاعل البلمرة المتسلسل المتعدد والأرانب والموت المفاجئ