Bacteriological Studies on *Mannheimia haemolytica* Isolated from Rabbits Suffered from Wry Neck.

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Abstract

Rabbit farming is a growing industry to satisfy the great demand for food, particularly in developing countries. In this study, out of 450 samples collected from 150 rabbit cases suffering from wry neck, 100 from clinically diseased rabbits and 50 freshly dead ones with a history of wry neck. All cases were subjected to clinical examination, bacterial culture and polymerase chain reaction. 30 samples out of 450 samples were positive for *M. haemolytica* with an incidence of 6.6% mixed with *E.coli* (serotypes O78, O26 and O2) and *Staph aureus* with incidence of 3.3% and 2.2% respectively were concomitantly isolated. In-vitro antimicrobial susceptibility testing showed a wide range of multidrug resistance against penicillins and fluoroquinolones whereas colistin sulphate was the most effective antibiotic followed by chloramphenicol, neomycin and oxytetracycline. The findings of this study revealed that multidrug resistant bacteria constitute a serious problem in commercial rabbiteries in Egypt. PCR technique was done on original samples and it appeared that conventional PCR technique more sensitive than the conventional microbiological technique (CMT). Effective control measures are required to mitigate the economic impact on the rabbit industry and to prevent possible public hazards.

Keywords: Rabbit; Bacterial infections wry neck; *Mannheimia haemolytica*; Antimicrobial resistance

Introduction

Rabbit industry is one of the fastest growing livestock industries worldwide. Domestic rabbits are raised for meat production, fur, laboratory use and/or as pets. Farming of rabbits for meat production is supported by the continuously growing demand for protein of animal origin ***(Lebas et al, 1997).*** Global production of rabbit meat has been substantially

increased from 0.9 million ton in 1990 to more than 1.7 million tons in 2011 and the greatest increases occurred in the last few years ***(FAO, 2013).*** Generally, rabbits have high productivity in terms of breeding, fast growth rate, good carcass quality and superior nutritive properties ***(Dalle Zetto and Szendro, 2011).*** Although the rabbit industry is expected to grow at a faster rate in the coming years, this industry is facing challenges in leveraging this growth rate mainly due to infectious diseases ***(Langan et al, 2000 and Lennox and Kelleher, 2009).*** Bacterial infections in rabbits can negatively influence the body condition and cause high mortality especially during the early months of life. Wryneck (Torticollis) is a fatal condition in rabbits where the head or the neck becomes twisted to one side which may be preceded (or accompanied) with otitis, unsteady gait, incoordination, rolling or movement in circles ***(Harcourt Brown, 2002).*** Torticollis is multifactorial in origin and likely results from bacterial infections, encephalitozoonosis, cerebral larva migrans or trauma to the central nervous ***system (Kunstyr and Naumann, 1985 and Vernau et al, 2007).*** *Mannheimia haemolytica* (*M. haemolytica*) is a Gram negative, bipolar, hemolytic aerobic bacteria which was considered a member of normal flora of the upper respiratory tract of rabbits ***(Ajuwape and Aregbesola, 2002).*** Recently, *M. haemolytica* was isolated from respiratory tract problems of different birds and rabbits ***(Abdel-Aziz, 2000; Vipasha et al, 2004 and Rougier et al, 2006)***. Although it is commonly seen and most commercial rabbits affected with this condition are culled, little is known about the prevalence of bacterial agents (except for *Pasteurella Multocida*) associated with wry neck in domesticated rabbits. Importantly, treatment of wry neck in rabbits requires long term use of high doses of antibiotics which is neither efficient nor cost-effective in commercial rabbit production ***(Patton et al, 2008)*** and may accelerate the emergence of antimicrobial resistance which is a major problem worldwide both in veterinary and human medicine ***(Garcia-Alvarez et al, 2012).*** Furthermore, several zoonotic bacteria of rabbits origin can be transmitted either directly (i.e., through bites, consumption of foods) or indirectly (i.e. through exposure to excretions) to humans with serious health consequences ***(Mani and Maguire, 2009; Rodriguez et al, 2006; Sampasa et al, 2012 and Swennes et al, 2012).*** In under-resourced countries like Egypt, rabbit production is increasing to secure food and to increase farmer's incomes.

Therefore the goal of this study was planned to isolate and characterize *Mannheimia haemolytica* associated mainly with wry neck in rabbits in

Egypt. Testing the antimicrobial sensitivity of the isolated M. *haemolytica* to different antimicrobial agents and comparison between the sensitivity of standard method of isolation and PCR technique.

Materials and methods

Samples

A total of 450 samples were collected aseptically from rabbits of different ages from private farms of different governorates of Egypt. The surveillance was targeted mainly to rabbit farms with a history of wry neck (100 cases from clinically ill rabbits and 50 cases from recently dead ones) at different governorates in Egypt. Collected samples included nasal and ear swabs from diseased rabbits and internal organs (heart, liver, lungs, brain and/or bone marrow) from recently dead rabbits. Post-mortem examination was carried out for the dead rabbits.

Bacteriological examination

All the collected samples were pre-enriched in buffered peptone water (Oxoid Ltd, UK) and incubation at 37°C for 24 hours. Thereafter, a loopful of each broth culture was inoculated onto blood agar, MacConkey agar, XLD agar (Oxoid Ltd, UK), Eosin methylene blue agar and Baird parker agar (HiMedia Laboratories, Mumbai, India) then incubated at 37 °C for 24 hours. Selected colonies were identified morphologically, microscopically and biochemically according to the standard protocols ***(Holt et al, 1994).*** In addition, pathogenicity test for each isolate of *M. haemolytica* 2 mice were inoculated intra-peritoneal with 0.2 mL of overnight incubated broth and heart blood from dead mice was smeared and stained with crystal violet for detection of Pasteurella bipolarity and further used for re-isolation and identification of *M. haemolytica*.

Serotyping of E. coli isolates:

E.coli isolates were serotyped by slide agglutination test according to ***(Lee et al, 2009)*** using standard *E. coli* antisera **(Sifin and Denka Seiken Comp**.).

Antibiotic sensitivity tests

Antibiotic susceptibility testing of *M. haemolytica* isolates was performed using disk-diffusion method as described by ***(Cruickshank, 1975 and NCCLS, 2002)*** against a range of 12 antibiotic discs purchased from Oxoid (Basingstoke, UK): ampicillin (10 µg), amoxicillin (25 µg), colistin sulphate (10 µg), ciprofloxacin (5 µg), danofloxacin (5 µg), enrofloxacine (5 µg), gentamycin (10 µg), oxytetracycline (30 µg), neomycin (10 µg), chloramphenicol (30 µg), penicillin (10 IU) and/or sulphamethoxazole – trimethoprim (SXT) (25 µg). Inhibition zones were measured and interpreted as resistant (R), intermediate (I) or susceptible (S) as shown in table (6).

Statistics

Results of antibiotic sensitivity were compared by using a Chi-

square test and one-way analysis of variance (ANOVA) with Statistical Package for the Social Sciences version 15 (SPSS) software for Windows. Values were considered to be statistically significant when P-values were less than 0.05

Polymerase chain reaction (PCR)

DNA extraction from all samples was performed using the QIAamp DNA Mini kit (Qiagen GmbH, Germany) according to the manufacturer's recommendations with minor modifications. Briefly, 200 µl of the sample suspension was incubated with 20 µL of proteinase K and 200 µL of lysis buffer at 56 °C for 10 minutes. After incubation, 200 µL of 100% ethanol was added to the lysate. After two successive washing steps, DNA was eluted in 100 µL of the provided elution buffer. Specific oligonucleotide primers (Midland Certified Reagent Company, TX, USA) were used for partial amplification of virulence-associated genes found in pathogenic bacteria namely DNA methyltransferases (mod) gene of M. haemolytica ***(Deressa et al, 2010).*** Amplification reactions were performed in a volume of 25 µl/reaction containing 12.5 µl of Emerald Amp PCR Master Mix (Takara, Shuzo, Kyoto, Japan) and the PCRs were done in a T3000 Biometra thermal cycler. The resulting amplicons were separated by 1-2% agarose gel electrophoresis (Applichem GmbH, Germany). To determine the size of DNA fragments, 100 bp or 100 bp plus DNA Ladder (Qiagen GmbH, Germany) was used. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1: *Types and numbers of examined samples*

Type of sample	No.of sample
Ear swabs	100
Nasal swabs	100
Brain	50
Bone marrow	50
Lung	50
Liver	50
Heart	50
Total	450

Table 2: *Oligonucleotide Primers used for PCR amplification*

Primer	Primer sequence 5´- 3´	Gene	Annealing temp.	Amplified fragment	Reference
Rpt2 F	GTTTGTAAGATATCCCATTT	*mod*	48 °C	1022 bp	Deressa et al., 2010
Rpt2 R	CGTTTTCCACTTGCGTGA				

Results and discussion

Bacterial infections cause severe economic losses in rabbit industry particularly in developing countries. In this study we describe isolation, identification and antibiotic susceptibility of bacteria isolated from commercial rabbits in Egypt. In this study bacteriological examination of 450 rabbit samples collected from 150 rabbit cases (100 clinically ill rabbits and 50 from recently dead ones) at different governorates in Egypt had wry neck (torticollis) as a common clinical sign in addition to rhinitis, conjunctivitis, diarrhoea and/or sudden death with a history of high morbidity and mortality narrated by the owner, in addition to, postmortem examination of the dead rabbits showed septicaemia, pinpoint necrotic foci in liver, pneumonia and/or pale kidneys, revealed 6 .6% positive isolation of *M. haemolytica*. (Table3). Similar results were recorded by ***Vipasha Kapoor et al (2004)*** who reported the isolation of *P. haemolytica* (*M. haemolytica*) from nasal swabs and pneumonic lungs from healthy and diseased rabbits suffering from respiratory disorders with an incidence of 0.39%. Previous isolation of those organisms from pet and laboratory rabbits suffering from respiratory and/or enteric diseases have been frequently reported ***(Deeb and Carpenter, 2004)*** but relatively little is known about their association with torticollis in commercial rabbiteries. Also, ***Rougier et al (2006),*** could isolate *M. haemolytica* from rabbit cases suffering from upper respiratory tract disorders with an incidence of 0.9%. in addition to ***Badr et al (2008)*** who recorded that bacteriological examination of 525 rabbit samples collected from 225 rabbit cases suffering from conjunctivitis and different respiratory disorders (nasal discharges, coughing, sneezing, difficult breathing, etc.), revealed 5.5% positive isolation of *M. haemolytica*. The increase in the incidence of isolation of *M. haemolytica* in the present work might be correlated to concerning the isolation only from diseased

rabbit cases suffering from wry neck with other clinical signs. Single infection with *M. haemolytica* was recorded in twenty cases while ten cases were mixed-infected with *E. coli* and *S. aureus* (table 4). This results correlated with that obtained by ***Mahmoud et al (2005)*** detected *E. coli, Diplococcus pneumonia* and *Bordetella bronchiseptica* associated with Pasteurella infection of rabbits. Also, ***Rougier et al (2006),*** detected polymicrobial infections in rabbit samples collected from rabbits suffering from upper respiratory tract infections, particularly the combination of *P. multocida* and *B. bronchiseptica* in 28% of pet rabbits. In addition to, ***Badr et al (2008)*** mentioned that the *M. haemolytica* isolates were found mixed with other bacterial agents from the examined samples including: *Pasteurella multocida* (58.6%), *Bordetella bronchiseptica* (23.4%), *Klebsiella pneumonia* (13.5%), *Staphylococcus aureus* (3.6%) and *Pseudomonas species* (0.9%).

In contrast to our findings, higher prevalence of S. aureus (9.6%) and E. coli (9.6%) than *M. haemolytica* (0.9%) was reported in pet rabbits suffering from snuffles in France and the United Kingdom ***Rougier et al (2006)***. Surprisingly, the frequent E. coli serotype in this study was serotype O78 that has been frequently associated with extra-intestinal disorders, such as septicaemia, airsacculitis and pneumonia in poultry, livestock, and humans ***(Moulin et al, 2007).*** On the contrary, serotypes O26 and O2 have been isolated from feces and cecal contents of healthy and diarrheic rabbits ***(Blanco et al, 1996; Blanco et al, 1994 and Peeters et al, 1988)*** and some members of serotypes O26 could transmit to humans causing diarrhea or hemorrhagic colitis ***(Pollock et al, 2011).***

Results of in-vitro sensitivity testing showed that 74 to 96% of the isolated bacteria were significantly resistant (p values <0.0001) to penicillins (penicillin, ampicillin and amoxicillin), fluoroquinolones (ciprofloxacin, enrofloxacin and danofloxacin), gentamycin and/or SXT as shown in (Table 6). Colistin sulphate was found to be the most effective antibiotic against 86% (n=47/55) of the tested bacteria followed by chloramphenicol (80%, n= 44/55), neomycin (69%, n=38/55) and oxytetracycline (62%, n=34/55). Results of in-vitro sensitivity testing disagree with those reported previously by other research workers where penicillin, gentamycin and SXT were effective against different bacterial isolates in laboratory rabbits ***(Scheld, 1983)*** and similar findings were reported from pet rabbit respiratory disease [12]**.** Nevertheless, ***Badr et al (2008)*** who recorded that Antibiotic sensitivity testing of the isolated bacterial agents revealed high susceptibility to ciprofloxacin and

norfloxacine followed by gentamycin and colistin sulphate. Our results are partially in accordance with previous study which showed that *M. haemolytica* were resistant to ampicillin, SXT, gentamicin and ciprofloxacin but were sensitive for chloramphenicol and amoxicillin/clavulanic acid ***(Poeta et al, 2010).*** It is worth pointing out that laboratory and pet rabbits may be not exposed to (or under pressure of) antibiotics as much as the commercial rabbits. The observed multidrug resistance in this study is probably as a result of the massive misuse/overuse of antibiotics for treatment of diseases in rabbits in Egypt.

Results of PCR technique revealed that 31 out of 150 (21%) samples were positive by PCR. While, 30 out of 150 (20%) were positive for *P. haemolytica* by conventional method of isolation. (Figure 1). The results of the current study showed also that the PCR was more sensitive than the culture method (Table 7) which is in accordance with previous studies ***(Ennaji et al, 2009 and Mason et al, 2001).*** Nevertheless, because of the insufficiency of the data, the poor rate of isolation due to prior treatment of rabbits with antibiotics could not be ruled out.

Table 3: *Incidence of isolation of M. haemolytica from rabbit samples***.**

Samples		Total No.	*Total Isolates of M. haemolytica*		Single infection		Mixed infection	
source	organ		No.	%*	No.	%**	No.	%***
Disease d rabbits	Ear swabs	100	15	15	6	30	9	90
	Nasal swabs	100	5	5	4	20	1	10
Dead rabbits	Brain	50	4	8	4	20	0	0
	Bone marrow	50	0	0	0	0	0	0
	Lung	50	4	8	4	20	0	0
	Liver	50	2	4	2	10	0	0
	Heart	50	0	0	0	0	0	0
		450	30	6.6	20	66.7	10	33.3

* *Percentage according to the number of each organ*
** *Percentage according to the number of single infection*
*** *Percentage according to the number of mixed infection*

Table 4: *Bacterial agents other than M. haemolytica isolated from rabbits*

Bacterial isolates	Single		mixed		Total	
	No.	%*	No.	%*	No.	%**
M. haemolytica	20	66.7	10	33.3	30	54.5
E. coli	7	46.7	8	53.3	15	27.3
Staph. aureus	0	0	10	100	10	18.2
Total	27	77.6	28	22.4	55	100

**Percentage according to total number of the isolated species*
*** Percentage according to total number of the isolates.*

Table 5: *Serotyping results of E. coli isolated from diseased rabbits*

Serotype	No. of isolates	%*
O2	2	13.3
O26	3	20
O78	7	46.7
untypable	3	20
Total	15	100

**Percentage according to total number of the isolated E. coli*

Table 6: *Result of antibiogram of M. haemolytica isolated from rabbits*

Antimicrobial agent	*M. haemolytica*			*E. coli*			*Staph. aureus*		
	R.*	I.	S.	R.	I.	S.	R.	I.	S.
Ampicillin (10µg)	13	2	0	9	1	0	5	0	0
Amoxicallin(25 µg)	14	1	0	9	1	0	4	1	0
Chloramphenicol(30 µg)	1	14	0	8	2	0	0	5	0
Ciprofloxacin(5 µg)	13	2	0	8	2	0	4	1	0
Colistin sulphate(10 µg)	2	4	9	1	9	0	1	4	0
Danofloxacin(5 µg)	13	2	0	8	2	0	5	0	0
Enrofloxacin(5µg)	11	4	0	9	1	0	5	0	0
Gentamycin(10 µg)	12	3	0	8	2	0	4	1	0
Oxytetracycline(30 µg)	12	3	0	2	8	0	4	1	0
Neomycin(10 µg)	3	12	0	9	1	0	1	4	0
Pencillin(10 µg)	14	1	0	10	0	0	4	1	0
Sulphamethoxazole-trimethoprim	14	1	0	10	0	0	1	4	0

**R. =resistant, I. = intermediate, S. = sensitive*

Table 7: *comparison between the use of SMT and PCR for detection of M. haemolytica*

Bacterial agent	SMT*		PCR	
	No	%**	No	%**
M. haemolytica	30	20	31	21

*SMT= standard microbiological technique
**Percentage according to total number of samples.

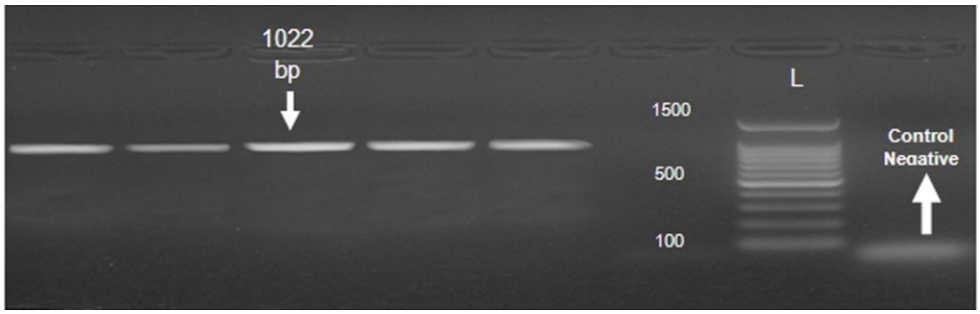

Fig 1: *Agarose gel electrophoresis showing positive amplification of 1022bp fragment using PCR with specific primer of mod gene of M. haemolytica*

CONCLUSION

It could be concluded that *M. haemolytica* is a potent pathogen that might threaten rabbit flocks as the microorganism can complicate other bacterial pathogens in rabbits which lead to increase the severity of infection. So, efforts should be paid to control the infection by applying strict hygienic measures of biosecurity in rabbit farms in association with good management and avoid exposure to stress factors which all take apart in controlling the infection in rabbit colonies. Taken together, multidrug resistant *M. haemolytica* are common in torticollis in rabbits. The wide range of drug resistance described here is a real challenge for rabbit industry and may be considered a serious public health problem. Further investigations should continue to characterize the antibiotic-resistance genes and the epidemiology link between rabbits and other farm animals and human. To reduce the emergence of multidrug resistant bacteria (1) public health awareness and hygiene measures should be the first line of defence against infectious diseases in rabbit's farm. (2) Autogenous vaccines may be a worthwhile approach particularly in endemic regions. (3) Although practically difficult, chemotherapy should be the last resort of treatment and it is strongly

recommended to make an antibiogram for the isolated bacterium before starting therapy.

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الملخص العربى

دراسات بكتيرولوجية على مانهيميا هيموليتكا المعزولة من الارانب التى تعانى من التواء الرقبة

ايمان محمد فرغلى ،انجى أحمد حامد ،هبه رشدى و أحمد محمد عبد الرحمن عرفان

المعمل المرجعى للرقابة البيطرية على الانتاج الداجنى – معهد بحوث صحة الحيوان – مركز البحوث الزراعية – وزارة الزراعة – الدقي – الجيزة.

نظرا لان التواء الرقبة فى الارانب يعد من المشاكل المرضية الكبرى فى مزارع الارانب والتى ينتج عنها اما النفوق او التخلص نهائيا من القطيع لذلك لزم اجراء دراسات على أحد المسببات البكتيرية لتلك المشكلة وهو ميكروب المانهيميا هيموليتكا. لذلك تم فحص ٤٥٠ عينة من الارانب المريضة والنافقة حديثا من مزارع الارانب المختلفة فى مصر وتم اجراء الصفة التشريحية وتجميع العينات اللازمة للعزل البكتيرى. اوضحت الفحوص البكتيريولوجية ان ٣٠ عينة ايجابى لعزل مانهيميا هيموليتكا بنسبة ٦,٦٪. وقد تم عزل بعضها مصحوبا بانواع أخرى من البكتريا مثل الايشيرشيا كولاى والاستاف أورويس بنسبة ٣,٣٪ و ٢,٢٪ على التوالى . وقد تم تصنيف عترات الايشريشيا كولاى سيرولوجيا. وتم اجراء اختبار الحساسية للعترات المعزولة باستخدام المضادت الحيوية المختلفة ووجد انها مقاومة لمعظم المضادت الحيوية المستخدمة خاصة مجموعة الفلروكلينولينات مثل السبروفلوكساسين، الانروفلوكساسين، الدانوكساسين ولكنها حساسة للكولستين سلفات ثم النيوميسين والكلورمفنيكول ثم السلفاميثوكسازول ترايميثوبريم. تم اجراء اختبار انزيم البلمرة المتسلسل على العينات ووجد انها اكثر حساسية من طرق العزل البكتيرية التقليدية. كما يتحتم اتباع قواعد الامان الحيوى للتخفيف من الاثر الاقتصادى على صناعة الارانب وللوقاية من الاخطار المحتملة العامة.