INCIDENCE OF MANNHEIMIA HAEMOLYTICA IN RABBITS FARMS IN EGYPT

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SUMMARY
Snuffles disease is a major problem in rabbits caused by Pasteurella multocida. Pasteurella multocida is well established to be the major etiological agent of the disease although Mannheimia haemolytica has also been incriminated in many acute outbreaks. In the current study, 200 samples were collected from freshly dead (n = 49) and diseased (n = 151) rabbits of different ages from private farms of Egypt. The rabbits were suffering from various signs of respiratory disorders (nasal discharges, sneezing, coughing, conjunctivitis and other respiratory disorders. All cases were subjected to clinical examination, morphological, biochemical identification and molecular characterization of the isolates using PCR. 36 out of 200 samples (18%) were positive for M. haemolytica by conventional bacteriological methods. Using PCR, 29 out of 36 isolates (80.6%) had Ssa gene M. haemolytica. The current study recommends field consideration from veterinarian and researchers to the respiratory affection and external lesions of rabbits and the Mannheimia haemolytica contribution.

Keywords:
Bacterial infections, Mannheimia haemolytica, Pasteurella, Rabbit.

Introduction:
Commercial rabbit's husbandry is an important industry for meat, fur and leather. Respiratory infections of rabbits are considered a serious problem facing rabbitaries due to high mortalities, increased medication costs and body weight losses (Langan et al., 2000). Mannheimia haemolytica is a Gram negative, bipolar, hemolytic aerobic bacterium 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-Kapoor et al., 2004 and Rougier
et al., 2006). 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 Zotte and Szendro, 2011). Few studies were concerned with the role of this pathogen in respiratory tract infections of rabbits. Hence the goal of this study was to determine the incidence of *M. haemolytica* in the respiratory tract of rabbits, by clinical examination, morphological and biochemical identification of the isolates and molecular characterization using Conventional PCR.

**MATERIALS AND METHODS**

**Sample collection:**
A total of 200 samples were collected from freshly dead (*n* = 49) and diseased (*n* = 151) rabbits (Table 1) of different ages from private farms of different governorates of Egypt. The samples were collected aseptically from rabbits suffering from various signs of respiratory disorders (nasal discharges, sneezing, coughing, conjunctivitis and other respiratory disorders. The collected samples included nasal swabs, ear swabs, skin, brain, kidney, lungs, heart, liver and bone marrow, were subjected to bacteriological and biochemical identification.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diseased rabbits: <em>n</em> = 151</td>
<td></td>
</tr>
<tr>
<td>Swabs</td>
<td>106</td>
</tr>
<tr>
<td>Swabs</td>
<td>34</td>
</tr>
<tr>
<td>Abscesses</td>
<td>11</td>
</tr>
<tr>
<td>Freshly dead rabbits: Organs</td>
<td>49</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
</tr>
</tbody>
</table>

**Bacteriological examination:**
All the collected samples were pre-enriched in buffered peptone water (Oxoid Ltd, UK) and after incubation at 37°C for 24 hours they were inoculated simultaneously on Dextrose starch agar, sheep blood agar and MacConkey agar (Oxoid Ltd, UK), then all plates were incubated
aerobically at 37°C for 24 hours. Thereafter, a Selected colonies were identified morphologically, microscopically and biochemically according to the standard protocols (Quinn et al., 1994). In addition, for *M. haemolytica* isolate two mice were inoculated intra-peritoneal with 0.5 ml of overnight incubated broth and heart blood from dead mice was smeared and stained with Gram's stain for detection of pasteurella bipolarity and further used for re-isolation and identification of *M. haemolytica*.

**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 gene found in pathogenic bacteria namely PHSSA F,Ssa gene (Table 2) and 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). 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 analysed through computer software.

**Table (2): Oligonucleotide primers sequences.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Primer sequence (5′-3′)</th>
<th>Length of amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHSSA F</td>
<td><em>M. haemolytica</em></td>
<td>5′-TTC ACA TCT TCA TCC TC-3′</td>
<td>500 bp.</td>
<td>Hawari et al. (2008)</td>
</tr>
<tr>
<td>PHSSA R</td>
<td><em>Ssa</em> gene</td>
<td>5′-TTT TCA TCC TCT TCG TC-3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

The examined rabbits in this study had bronchopneumonia, middle ear infections, abscessation and/or sudden death with a history of high morbidity and mortality narrated by the owner. Postmortem examination of the dead animals showed septicemia, pinpoint necrotic foci in liver, pneumonia and/or pale kidneys. The bacteriological examination indicated that out of 200 samples 36 (18%) samples was positive for *M. haemolytica* by culture and confirmed by PCR in 29 (80.5%) out of 36 (Table 3). Similar results were recorded by Badr *et al.* (2008) who reported the isolation of *M. haemolytica* from nasal swabs, liver, lung, bone marrow, trachea and heart revealed 29/525 (5.5 %) positive isolation of *M. haemolytica*. Similar results were recorded by Vipasha-Kapoor *et al.* (2004) who reported the isolation of *M. haemolytica* from nasal swabs and pneumonic lungs from healthy and diseased rabbits suffering from respiratory disorders with an incidence of 0.39%. Also, Rougier *et al.* (2006) isolated *M. haemolytica* from rabbit cases suffering from upper respiratory tract disorders with an incidence of 0.9%. Similar results were recorded by Farghaly *et al.* (2013) who detected *M. haemolytica* from nasal swabs, heart, liver, brain and bone marrow from diseased rabbits with a history of torticollis with incidence of 24 %. 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 respiratory disorders. All rabbits infected with *M. haemolytica* shown different signs of pasteurellosis while postmortem examination of dead rabbits shown severe congestion of internal organs, hemorrhages in the tracheal mucosa and pneumonic lungs. These results completely agree with that obtained by Heng *et al.* (1996) who reported the ability of lipopolysaccharides isolated from *M. haemolytica* A2 to induce clinical signs and lesions similar to those of pasteurellosis in rabbits. Some members of genus *Mannheimia* produce number of substances that are associated with the virulence of this group of microorganisms (Tefera and Smola, 2001). Among important substances produced are the capsule, lipopolysaccharides, iron-regulated outer membrane proteins, toxic outer membrane proteins, adhesions, leukotoxin and enzymes namely hyaluronidase and neuraminidase (Biberstein and Dwight, 1999). *M. haemolytica* are commensally resident in the respiratory tract of healthy ruminants and are capable of causing infection in animals with compromised pulmonary defense system due to some predisposing factors such as stress. Stress is an intrinsic condition that was consistently
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reported to increase the susceptibility to various types of infectious diseases in man and animals (Stephens, 1980; Biondi & Zannino, 1997). However, the effect of stress is more evident with respiratory tract infections in which pneumonic pasteurellosis may well provide the most appropriate example in veterinary medicine. Stress may either be psychological as induced by fear, restraint, handling or physical, resulting from hunger, thirst, fatigue or thermal extremes (Grandin, 1997). Stress cannot be measured grossly in an individual animal, yet, a number of clinical and biochemical parameters such as elevated body temperature, increased heart rate, decreased body weight and increased levels of plasma cortisol, glucose, free fatty acids, urea and betahydroxybutyrate rate were generally regarded as useful indicators (Knowles et al., 1995; Morton et al., 1995; Warriss et al., 1995). The reaction of animals to stress is rather variable even within individual animals of the same species. The role of stress in the natural incidence of pneumonic pasteurellosis was clearly evident by the fact that the disease onset is mainly associated with sudden exposure to stressful situations created by adverse physical, environmental or climatic conditions. The most common examples of these include extremely hot or cold weather with high levels of humidity, overcrowding in a limited space, poor ventilation, bad management and rough handling (Thomson et al., 1975; Slocombe et al., 1985; Radostits et al., 2000). Previous or combined infection with certain respiratory viruses was commonly found to increase the susceptibility of farm animals to secondary bacterial pneumonias (Carter, 1973; Al-Darraji et al., 1982; Cutlip et al., 1993; Brogden et al., 1998; Hodgson et al., 2005). In addition to the previously mentioned predisposing factors, a number of other unrelated conditions such as selenium deficiency, mycotoxins, inhalation of foreign material and obstruction of pulmonary airways were also reported to have a predisposing role in the incidence of pneumonic pasteurellosis in susceptible animals (Reffett et al., 1985; Pfeffer, 1988). Experimental evidence also indicated that the susceptibility to M. haemolytica and P. multocida infections was significantly increased in laboratory and farm animals by the repeated administration of injectable or dietary iron compounds (Al-Sultan and Aitken, 1984; Ali, 1999; Mohamed, 2002). The increased virulence of Mannheimia and other Pasteurella species by iron compounds was primarily attributed to the vital role of iron as a growth-promoting factor for unicellular microorganisms.
Conclusion:
It could be concluded that *M. haemolytica* is a potent pathogen that might threaten rabbit flocks as the microorganism can complicate other respiratory bacterial pathogens in rabbits which lead to increase the severity of respiratory 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 a part in controlling the infection in rabbit colonies.

Acknowledgements:
The authors are grateful to all colleagues and co-workers in the National Laboratory for Veterinary Quality Control on Poultry Production, Dokki-Giza, Egypt for their valuable support during the study. The authors are grateful to farmers and field veterinarians for their active participation in this study.

Table (3): Relation between isolated microorganism and different samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Positive PCR</th>
<th>Positive isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Nasal swabs</td>
<td>9</td>
<td>31.1</td>
</tr>
<tr>
<td>Ear swabs</td>
<td>6</td>
<td>20.7</td>
</tr>
<tr>
<td>Lung</td>
<td>4</td>
<td>13.8</td>
</tr>
<tr>
<td>Liver</td>
<td>3</td>
<td>10.3</td>
</tr>
<tr>
<td>Heart</td>
<td>2</td>
<td>6.9</td>
</tr>
<tr>
<td>Brain</td>
<td>3</td>
<td>10.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>2</td>
<td>6.9</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>100</td>
</tr>
</tbody>
</table>

Table (4): Comparison between the use of SMT and PCR for detection of *M. haemolytica* isolated from rabbits.

<table>
<thead>
<tr>
<th>SMT</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>%</td>
<td>%</td>
</tr>
</tbody>
</table>

SMT= standard microbiological technique

Percentage according to total number of samples
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Fig. (1): Agarose gel electrophoresis showing positive amplification of 500bp fragment using PCR with specific primer of Ssa gene of M. haemolytica.
Lane L: ladder, lane -: negative control, lane +: positive control and lanes 1-36 are the isolates.

REFERENCES


