



Pasteurella multocida in Cows: Identification of the Isolates by VITEK2 System and Detection of Toxigenic Strains by One-step ELISA

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Abstract | *Pasteurella multocida* is a pathogenic Gram-negative bacterium causing respiratory manifestation in cows and other animals resulting in great economic losses. This work aimed to identify the *P. multocida* using VITEK2 system and detect the toxigenic strains by one step enzyme-linked immune-sorbent assay (ELISA). For detection of *P. multocida*, 209 samples including 145 nasopharyngeal swabs (115 from cows with respiratory manifestation and 30 in contact apparent healthy animals) and 64 pneumonic lungs from dead cows were collected and examined. The recovered isolates were identified by both conventional methods and VITEK2 compact system. *P. multocida* isolates were investigated for toxin production by mouse lethality test and *P. multocida* toxin-specific monoclonal antibodies using ELISA. Out of 63 presumptive *P. multocida* colonies, 35 isolates (16.75%) were identified using the conventional methods and animal inoculation test; whereas, 43 isolates (20.57%) were confirmed by VITEK2 compact system and were subsequently tested for toxin production. Out of 43 positive *P. multocida* isolates, only 14 isolates (32.55%) were proved to be toxigenic by using a mouse lethality test; while with the help of a one-step enzyme immunoassay, 17 toxigenic *P. multocida* isolates (39.53%) were identified. VITEK2 compact system appeared as a fast and reliable method for identification and detection of *P. multocida*, and PMT ELISA was observed more sensitive than mouse lethality test.

Keywords | *P. multocida*, Nasopharyngeal swabs, VITEK2 system, one-step enzyme immunoassay

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INTRODUCTION

P. multocida is recognized as an important veterinary pathogen that causing diseases in different animal species and is the reason for many illnesses of high economic importance; such as, avian fowl cholera (Elalamy et al., 2020), hemorrhagic septicemia, enzootic pneumonia, and swine atrophic rhinitis (Ahmed et al., 2014). In humans, zoonoses often occur because of scratches, bites, or being licked by domestic pet animals, and the respiratory tract infection may also occur (Casolari and Fabio, 1988).

P. multocida is often linked to the extended cases of bovine respiratory disease. Decreased appetite is an early symptom of infection. Signs of serious infection including loss

of appetite, high fever, lowered head and ears, hard breathing, and muco-purulent nasal discharge. Infected animals will often suffer from a reluctance to move around, rapid shallow respiratory rate, and a moist cough. At this stage, if the animal is not treated, its lungs become irreversibly damaged, and it will die (Anderson and Rings, 2009).

Haemorrhagic septicaemia (HS) is a serious disease that affects buffaloes and cattle. It is caused by certain types of *P. multocida* that appear in southern Europe, Asia, Africa, and the Middle East (OIE, 2012).

P. multocida causes great losses, especially when animals are exposed to wet, chilly weather (Benkirane and Alwis, 2002). All animal ages are affected by *P. multocida*; however,

the most affected age group in cattle is from 6 months to 2 years. Mortality and morbidity vary between 50 and 100%, and the cured animals require a long convalescence. The immune status of the herd, whether due to natural infection or acquired by vaccination, greatly impacts morbidity. Naturally acquired immunity affects both morbidity and mortality to a great extent. The occurrence of the disease is decreased drastically by vaccination as well. However, the process through which animals recover from HS requires an extended period of time, which hinders the prevention of new outbreaks (Shahzad et al., 2020).

Differentiation between toxigenic and non-toxigenic strains of *P. multocida* is crucial for the detection and control of the diseases caused by the microorganism. Besides, health monitoring programs should be based on laboratory tests for the determination of toxigenic strains of *P. multocida* combined with clinical inspections of herds. Previous methods of differentiation have relied on the biological activities of *P. multocida* toxin, as lethality in mice, dermonecrotic impact on guinea pigs, and cyto-pathic effect on embryonic bovine lung (EBL) (Register and Brockmeier, 2019).

The traditional laboratory tests are based on the demonstration of the biological activity of toxin obtained from pure cultures of *P. multocida*. The advent of monoclonal antibodies to the toxigenic strains of *P. multocida* has led to the appearance of the *P. multocida* toxin (PMT) ELISA (one-step enzyme immunoassay) for rapid and simple identification. One of the main advantages of the PMT ELISA is assuring a specific and sensitive demonstration of PMT in any type of sample, including extracts of primary mixed cultures obtained from nasal swabs for instance (Chakraborty et al., 2017).

ELISA was employed by different researchers for detection of *P. multocida* toxins and reported a high percentage for *P. multocida* toxin using ELISA rather than other methods. Moreover, E1-Eragi et al. (2001) and Takada Iwoa et al., (2007) declared that ELISA had at least 86% sensitivity and 99% specificity. On the other hand, Filion et al. (1985) detected 100% sensitivity and specificity using ELISA (Zhong Peng et al., 2018).

This study was done for rapid identification of *P. multocida* strains from cows specimens using VITEK 2 system and to the detect it's toxin by one-step PMT ELISA.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN AND SAMPLING

All experimental protocols were in line with national and international standard and performed according to animal

ethical guidelines.

Nasopharyngeal swabs were collected from 115 cows suffering from respiratory manifestation and 30 from apparent healthy ones that were in contact with diseased animals in the Sharkia governorate in the winter season. A single swab was used for the nasal cavity and then placed in phosphate-buffered saline (a non-nutritive transport medium); and to avoid overgrowth of other bacteria, it is kept at 4-8°C during the transition that did not exceed 24 h.

Pneumonic lungs were collected from 64 newly dead cows that had a history of severe respiratory troubles, nasal muco-purulent discharge, and pyrexia. Using sterile plastic bags, samples were packed separately, labeled, and transferred in an ice box to the laboratory.

BACTERIOLOGICAL EXAMINATION

Nasopharyngeal swabs were inoculated into casein sucrose yeast (CSY) broth for 6-8 h, and then a loopful was cultivated onto casein sucrose yeast (CSY) agar, sheep blood, agar, and MacConkey agar (Oxoid, England).

Organ specimens were cultured directly onto the previously mentioned media. All agar plates were incubated at 37°C for a minimum of 48 h. The suspected colonies were picked up for morphological and biochemical identification (Koneman et al., 1994; Quinn et al., 2002) as a traditional method of identification.

DETECTION OF PATHOGENICITY BY ANIMAL INOCULATION OF ISOLATED *P. MULTOCIDA*

P. multocida suspected isolates were tested for pathogenicity using Swiss Webster white mice (n=5; weighing about 18-22g) obtained from Animal Health Research Institute, Dokki, Giza, Egypt, for each isolate. All important principles in laboratory animal management and ethics of using the animal for experimentation as well as animal welfare protocols were adopted. All 50 mice were injected (I/P) by 0.1 ml of a bacterial suspension of (1.5×10^8 cfu). One mouse was kept as a control for each isolate and was injected I/P with 0.1 ml sterile normal saline. The mortality rate and post mortem lesions were carefully recorded. Re-isolation of inoculated strains was carried out from the heart blood of the dead mice, and blood films were thoroughly prepared and stained with Leishman stain (Bio-Diagnostic, Egypt) to show *P. multocida* features (Wessman, 1964).

IDENTIFICATION OF *P. MULTOCIDA* USING VITEK2 COMPACT SYSTEM

Suspension Preparation: A sterile swab was used to transfer the necessary colonies of pure culture to suspend in 3.0 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, PH 4.5 to 7.0). The turbidity was adjusted to the equivalent of a 0.5-

Table 1: Prevalence of *P. multocida* in apparent healthy, pneumonic, and newly dead cows using conventional methods and VITEK2 compact system

Animal status	Type of sample	No. of samples	Suspected colonies	No. of <i>P. multocida</i> isolates (%)	
				By conventional methods	By VITEK2 System
Apparently healthy	Nasopharyngeal swabs	30	9	2 (6.67)	3(10)
Diseased	Nasopharyngeal swabs	115	35	22 (19.13)	26 (22.61)
Newly dead	Pneumonic lung	64	19	11 (17.19)	14 (21.87)
Total		209	63	35 (16.75)	43 (20.57)

Table 2: Prevalence of toxigenic *P. multocida* in apparent healthy, pneumonic, and newly dead cows using mouse lethality test and PMT ELISA.

Animal status	Type of samples	No. of <i>P. multocida</i> Isolates tested	No. of toxigenic <i>P. multocida</i> isolates (%)	
			By mouse lethality test	By PMT ELISA
Apparently healthy	Nasopharyngeal swabs	3	0 (0)	1 (33.33)
Diseased	Nasopharyngeal swabs	26	10 (38.46)	11 (42.31)
Newly dead	Pneumonic Lung	14	4 (28.57)	5 (35.71)
Total		43	14 (32.55)	17 (39.53)

0.63 McFarland turbidity with a VITEK2 Densi-Check (biomerieux, France).

Inoculation: For identification of each isolate Gram-negative (GN) cards were inoculated with microorganism suspension. The card was identified by different 47 biochemical tests. The microorganism suspension test tubes was set into a special rack (cassette) and placed into a vacuum chamber station. The organism suspension was transferred into micro-channels that filled all the test wells after the vacuum had been applied and the air was re-introduced into the station.

Card Sealing and Incubation: Inoculated cards were passed by a process that cuts off the transfer tube and automatically seals the card before loading into the carousel incubator. All cards were incubated on a line at 35.5°C ± 1.0 °C for approximately 6 hours. During incubation, the cards were read every 15 min automatically. The final results were obtained and automatically printed within 6-8 hours. All used cards were automatically dispensed into a waste container (Biomerieux 2006; Sahar et al., 2014).

DETECTION OF *P. MULTOCIDA* TOXIN BY MOUSE LETHALITY BIOASSAY

Each *P. multocida* isolate was cultured onto blood agar and incubated for 24 h at 37°C. The culture was scraped from plates into phosphate-buffered saline (PBS) containing 6 mM EDTA and 3 mM Phenyl-methylsulfonyl fluoride (Sigma, Chemical Co.). The suspension was sonicated and centrifuged (1.500xg for 30 min) then the supernatant was filtered (pore size 0.22µm). Aliquots (0.5 ml) of sonicate was injected I/P in four mice (test mice). Four control mice were inoculated in parallel with heat-inactivated sonicate

(70°C, 30 min). Both groups of mice were observed for 48 h. If all the test mice died, the isolate was considered toxigenic (Lichtensteiger et al., 1996).

DETECTION OF *P. MULTOCIDA* TOXIN USING PMT ELISA KITS

One-step enzyme immunoassay for detection of *P. multocida* toxin antibodies was employed on the basis of commercially available PMT Kits (code no k000911-9 Oxoid, England) in which plate was coated with *P. multocida* toxin (PMT). The test procedure was adopted according to manufacturer instruction and at the end micro-wells were read photo metrically at absorbance of 450 nm. The OD value of the two negative and positive control wells was calculated (Foged et al., 1988).

RESULTS

Bacteriological investigation of 209 different samples revealed 63 suspected colonies of *P. multocida*. Identification using conventional methods showed isolation of 35 (16.75%) *P. multocida* isolates. Out of 145 nasopharyngeal swabs collected from apparent healthy (30) and diseased animals (115), 24 isolates (2 of them from apparently healthy and other 22 from diseased) were positive for *P. multocida*. Likewise, 11 isolates were obtained from 64 pneumonic lung samples that were collected from newly dead animals (Table 1). Investigation of the suspected *P. multocida* colonies (63) using the VITEK2 compact system revealed that 43 (20.57%) *P. multocida* isolates were recovered from 209 samples. Out of 145 nasopharyngeal swabs, 29 isolates (3/30 from apparently healthy and 26/115 from diseased cows) were positive for *P. multocida*. Similarly, 14 isolates were recovered from 64 pneumonic lung samples

(Table 1).

Detection of *P. multocida* toxin by mouse lethality test showed that out of 43 *P. multocida* isolates, 14 (32.55%) were toxigenic (Table 2). Detection of toxigenic *P. multocida* isolates was also done using PMT ELISA. The OD value of positive and negative control was 135 and 0.53 respectively. The results showed that out of 43 *P. multocida* strains, 17 (39.53%) were toxigenic (Table 2).

DISCUSSION

Pasteurella is a pathogen that infects cows' respiratory systems leading to bovine respiratory disease. *Pasteurella* is usually a secondary bacterial invader, *i.e.*, another disease, like a virus, first weakens the immune system, which paves the way for the *Pasteurella* to infect the diseased animal. *P. multocida* is a common commensal or opportunistic pathogen found in upper respiratory tract of most cattle; however, it causes serious diseases when infection reach the lower respiratory tract. The transmission of infection with *Pasteurella* spp. is through direct contact, or by ingestion of contaminated feed or water contamination by nasal and oral discharges from infected cattle (Smith, 2009).

Preliminary identification of *P. multocida* isolates was carried out according to standard biochemical tests as described earlier (Koneman, 1994; Quinn et al., 2002). Detection of *P. multocida* by conventional methods was at a rate of 16.75%; while it reached 20.57% by VITEK2 compact system. VITEK2 is a new automated identification system accompanied by identification cards that give rapid and reliable identification as mentioned by (Seol et al., 2002) and (Chun-Hong Du et al., 2020) that used VITEK 2 system for rapid identification of *P. multocida* isolates from a large scale fatal outbreak among wild rodents in south western China.

This incidence (20.57%) was nearly similar to what was reported previously in Egypt by (Enany et al., 2012; Khadr 2005) who isolated *P. multocida* with an incidence rate of 19.23% and 22.73% from pneumonic calves respectively. While a higher incidence of *P. multocida* (34.3, 39.2, and 34.4 %) from the lung of slaughtered calves were obtained in Egypt in earlier studies by Elshemy and Abd-Elrahman, (2013), Abd-ELkaleik, (2013) and EI-Jackee, et al. (2015) respectively. The lower incidence was reported by several authors in Egypt (Sedeeq and Thabet, 2001; Defra, 2006; Sayed and Zaytoun, 2009) who isolated *P. multocida* in an incidence of 8.3, 15, and 15.89 % respectively.

Pneumonic Pasteurellosis is the main cause of severe respiratory tract infection in calves and cause great economic losses. *Pasteurella multocida* was singly isolated from 4.9%

of cases while it was mixed with other bacteria as *S. aureus*, *E. coli* and *Streptococcus* sp. with percentage of 4%, 1.2%, and 2.2% respectively. Authors further evaluated the antimicrobial susceptibility of *Pasteurella multocida* isolates that was showing high to fluoroquinolones and cephalosporins, on the other hand, highly resistance were obtained against tetracycline, penicillin and aminoglycosides (El-Seedy et al., 2019).

Now a day, the modern developed molecular and automated techniques are used in microbiological studies using multiplex PCR for molecular typing of capsular antigens of *Pasteurella multocida* and was simple, sensitive, rapid and reliable technique instead of serological techniques for identification of capsular antigen from calves and sheep in Egypt (Khalid S. Al-maary et al., 2017).

A confirmatory identification by VITEK2 compact system using Gram-negative card relies on biochemical tests, enzymatic activities and resistance. The identification card contains 47 different biochemical tests. Final results are available for approximately 10 hours (BioMerieux user guide, 2006). VITEK2 system is a novel automated tool used for the rapid identification of Gram-negative bacilli isolated from clinical specimens (Funke et al., 1998; Providencia joyanes et al., 2001; Farid et al., 2013).

VITEK 2 system is simple method for accurately detecting vancomycin resistant strains of enterococcus and can be used for antimicrobial susceptibility (Intetsu et al., 2004).

There was a variation in *P. multocida* detection using conventional traditional methods (16.75%) and the VITEK2 compact system (20.57%). The results of the current study are in agreement with other authors who found that VITEK2 gives reliable, rapid, and higher correct identification results than the conventional identification methods (Funke et al., 1998; Gavin et al., 2002; Ling et al., 2001; Chatzigeorgiou et al., 2011).

The 21.9% prevalence of *P. multocida* from the pneumonic lung of newly dead animals was referred to that mentioned by Quinn et al. (1994) who isolated *P. multocida* (19.7 %) from the deep pulmonary tissues of the diseased cases. This may refer to the presence of risk or stress conditions that induces favorable media in the lung enhance the invasion of *P. multocida* into deep tissues. Moreover, Karimkhani et al. (2011) illustrated that *P. multocida* was the major isolated bacteria from the lungs.

P. multocida was isolated from cows showed signs of respiratory disease and also from those without any manifestation (contact healthy). However, the herds suffering from respiratory disorders have high prevalence of toxigenic *P.*

multocida strains than non-toxigenic strains (Bowersock et al., 1992). Differentiation of toxigenic and non-toxigenic strains of *P. multocida* is vital for the precise diagnosis, cure, and avoidance of diseases caused by *P. multocida*. Numerous tests are used to identify toxigenic strains, including mouse lethality and in vitro cytotoxicity assays (Chakraborty et al., 2017). Thus, a distinctive test that does not require live animals and does not necessitate many efforts nor a long time is needed. The development of monoclonal antibodies to the dermonecrotic toxins of *P. multocida* has led to the appearance of the *P. multocida* toxin ELISA (one-step enzyme immunoassay) that is highly specific and sensitive in detecting this toxin.

These results are nearly similar to those analyzed previously in Denmark by (Foged et al., 1988) who found that out of 615 *P. multocida* isolates, 250 (40.65%) were toxigenic-strains by *P. multocida* toxin specific monoclonal antibodies in an enzyme-linked immune-sorbent assay (ELISA). A higher incidence was recorded by Matschullat et al. (1994) who found that 23 out of 42 strains were toxigenic for *P. multocida* by ELISA technique. Some *P. multocida* strains, which gave positive for one-step enzyme immunoassay were negative in the mouse lethality test. Out of 43 positive *P. multocida* strains, only 14 were proved to be toxigenic in an incidence of 32.55% using a mouse lethality test. These results are in agreement with that reported by Borowski et al. (2001).

The deviation occurs due to the intrinsic subjective evaluation of cytotoxicity assays, variances in the way of interpretation of many laboratories, and the existence of other substances in the supernatant fluids. Moreover, mouse-inoculation results were in some cases very difficult to interpret (Bowersock et al., 1992). It is clear that, with the monoclonal antibody test, the incidence of the toxin is discovered by a highly specific reaction to the toxin without any subjective consideration of the assay. The obtained findings are also in agreement with El-Eragi et al. (2001) and Takada-iwao et al. (2007) who declared that the sensitivity and specificity of the ELISA technique is 86% and 99% respectively. On the other hand, Filion et al. (1985) detected 100% sensitivity and specificity of the ELISA technique.

Recently, animal experiments for toxin detection have been increasingly replaced by laboratory modern techniques. Hence, ELISA tests can be highly recommended for toxin detection. The toxin detection of *P. multocida* for diagnostic purposes can be done without experimental animals (Schimmel et al., 1994). Chakraborty et al. (2017) declared that the main advantages of the PMT toxin ELISA compared to the lethality mouse test is the independence of laboratory animal facilities to handle several samples per

day.

Finally, there are several advantages to using PMT ELISA. It is economical, consumes less time, efforts, easy to applied and can be used for many samples at the same time, and produces objective results. Besides, one-step enzyme immunoassay is useful for checking herds for the prevalence of toxigenic *P. multocida* and evaluate the isolates for toxin production (Bowersock et al., 1992).

CONCLUSION

Most conventional methods are laborious and time-consuming, while the development of automated systems facilitate the identification of bacterial isolates. Current study demonstrated the presence of *P. multocida* in healthy, diseased as well newly dead animals using various techniques. VITEK2 compact system appeared as a fast and reliable method for identification and detection of *P. multocida* and PMT ELISA was more sensitive and specific in indicating toxigenic *P. multocida* strains than the mouse lethality test.

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CONFLICT OF INTEREST

The authors declare that there is no any conflict of interest.

AUTHORS CONTRIBUTION

H.S. and A. A. designed the experiments. All authors conducted the practical part of the work and analyzed the data. All authors wrote the draft and approved the final version of manuscript.

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