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ORIGINAL ARTICLE

Comparative studies for serodiagnosis of haemorrhagic septicaemia in cattle sera

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Abstract Haemorrhagic septicaemia caused by *Pasteurella multocida* is a major epizootic disease in cattle and buffaloes in developing countries with high morbidity and mortality rate. In the present study, a total of 88 *P. multocida* isolates were isolated from 256 nasopharyngeal swabs and lung tissues samples (34.4%) during the period from January, 2013 to March, 2014 from different governorates located in Egypt. Dead calves showed the highest percentage of *P. multocida* isolation followed by the emergency slaughtered calves, diseased calves then apparently healthy ones. These isolates were confirmed as *P. multocida* microscopically, biochemically by traditional tests and by API 20E commercial kit then by PCR. The percentages of positive serum samples using somatic antigen and micro-agglutination test at 1/1280 diluted serum were 10%, 54.49% and 0% in apparently healthy, diseased and emergency slaughtered samples, respectively whereas, the percentages using capsular antigen and indirect haemagglutination test were 40%, 60.89% and 60% in apparently healthy, diseased and emergency slaughtered samples, respectively. The ELISA showed the highest sensitivity for diagnosing *P. multocida* in apparently healthy, diseased and emergency

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slaughtered animals with percentages of 42%; 92.9% and 80%, respectively. The obtained results revealed that the ELISA using capsular antigen of *P. multocida* is a more sensitive and specific serological test for diagnosis of haemorrhagic septicaemia.

1. Introduction

Pasteurella multocida causes a serious disease in a wide range of avian, animal species and humans (Christensen and Bisgaard, 2006; Descamps et al., 2012) causing high economic losses in both developed and developing countries (Dziva et al., 2008). Haemorrhagic septicaemia (HS) is a major epizootic disease in cattle and buffaloes in many countries with high morbidity and mortality. Outbreak of HS among buffaloes and cattle could be due to the consumption of river water contaminated with infected carcasses with *P. multocida* beside aerosol route (Jesse et al., 2013). Several serological tests are used for the identification of the HS including slide agglutination test (Namioka and Murata, 1961), indirect haemagglutination test for capsular typing (Carter, 1955), the agar gel immunodiffusion test (Heddleston et al., 1972).

ELISA was a sensitive and specific test. The boiled-cell extract antigen was chosen as the best antigen prepared to be used in ELISA than whole bacterium antigen which is the least sensitive while, lipopolysaccharide antigen was more difficult to prepare and may be type specific (Klaassen et al., 1985). The aim of the present study was to evaluate the different serological tests in the diagnosis of haemorrhagic septicaemia.

2. Materials and methods

2.1. Samples

Under aseptic conditions, 206 nasopharyngeal swabs were collected from apparently healthy calves ($n = 50$) and from diseased calves suffering from septicemia and respiratory manifestations ($n = 156$). Also 50 lung samples collected from emergency slaughtered calves ($n = 35$) and dead calves ($n = 15$) were obtained from pneumonic calves during the period from January, 2013 to March, 2014 from different governorates located in Egypt. For serodiagnosis, a total of 221 blood samples were collected from apparently healthy animals ($n = 50$), diseased calves ($n = 156$) and emergency slaughtered animals ($n = 15$). All animals were chosen from different private farms in El-Behera and Kafer El-Sheikh governorates, Egypt which did not vaccinate animals against haemorrhagic septicaemia. The collected samples were transferred as soon as possible to the laboratory for bacteriological examination. Sera were separated from the blood samples for serodiagnosis of *P. multocida*.

2.2. Isolation and identification of P. multocida

The collected samples (nasopharyngeal swabs and lung tissues) were inoculated in Casein/sucrose/yeast broth (Oxoid) for 6–8 h. A loopful was cultivated on Casein/sucrose/yeast agar (CSY), blood agar and MacConkey agar plates then incubated at 37 °C for 48 h. The suspected colonies with culture characters of *P. multocida* were identified traditionally according to Quinn et al. (2002) using API 20 E tests (BioMérieu).

2.3. Polymerase chain reaction (PCR)

The procedure of PCR analysis was performed according to Townsend et al. (1998) and the instructor manual provided with kits (Qiagen) with some modification. A PCR reaction mixture (20 µl total volume) was prepared as follows: 3 µl nuclease free water, 10 µl Hot Start Taq plus master mix (2×), 2 µl Coral Load concentrate (10× optional), 1 µl forward species specific primer KMT1T7 (5′-ATCCGCTATTTACCC AGTGG-3′), 1 µl reverse primer KMT1SP6 (5′-GCTGTAA ACGAACTCGCCAC-3′) and 3 µl Extracted DNA. The PCR cycling profile consisted of one cycle for initial heat activation of 95 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and final cycle for 10 min at 72 °C. Then, the PCR products were visualized by agarose gel electrophoresis. Agarose gel electrophoresis was carried out according to Sambrook et al. (1989) to evaluate the amplified fragments using standard PCR markers and 100 bp ladder.

2.4. Pathogenicity test in mice (OIE, 2008)

Each isolate was inoculated in 3 mice intramuscularly by 0.1 ml of 24 h broth culture of *P. multocida* isolate. The turbidity of the broth was adjusted to McFarland's tube no 4. The mice were kept under observation for 72 h after inoculation, dead mice were necropsied and live mice were killed after 3 days and necropsied. Blood films were prepared and stained with Giemsa stain and examined for the presence of bipolarity.

2.5. Preparation of hyperimmune serum and P. multocida antigens

Hyperimmune serum was prepared in New-Zealand rabbits (2–3 kg) according to OIE, 2008 to be used as positive control in the serological assays. The somatic antigen from *P. multocida* strain was prepared in order to be used for micro agglutination test (MAT) and ELISA assays according to Namioka and Murata (1961). The capsular antigen from *P. multocida* strain was prepared in order to be used for indirect haemagglutination test (IHAT) and ELISA assays according to OIE, 2008.

2.6. Serodiagnosis assays

Micro agglutination test was applied in U-shape bottom plate using the prepared somatic antigen according to Shewen and Wilkie (1982). 1% sensitized sheep RBCs were prepared according to Carter (1955). Indirect haemagglutination test

Table 1 Occurrence of *P. multocida* in different samples of calves.

Source of the samples	Type of the samples	No. of the samples	Occurrence of *P. multocida*	
			No.	%
Apparently healthy animals	Nasopharyngeal swabs	50	10	20
Diseased animals		156	51	32.7
Total		206	61	29.6
Emergency slaughtered	Lung	35	15	42.9
Dead animals		15	12	80
Total		50	27	54
Total (256)		256	88	34.4

was applied in U-shape bottom microplate according to Sawada et al. (1982). ELISA test was performed according to the standard technique described by Takada-Iwao et al. (2007) using somatic antigen in the coating buffer and the same procedure was repeated with capsular antigen.

3. Results

All isolates had smooth, glistening, translucent and non-haemolytic colonies. There is no growth on MacConkey's agar medium. All isolates killed mice within 24 to 36 h post inoculation. Leishman's stained smears prepared from heart blood of dead mice revealed bipolar organisms and *P. multocida* were re- isolated on CSY agar. The *P. multocida*-specific PCR assay based on KMT1T7 and KMT1SP6 primers gave a positive signal by amplicon of approximately 460 bp with all *P. multocida* isolates.

A total of 88 *P. multocida* isolates were isolated from the collected samples (34.4%). Dead calves showed the highest percentage of *P. multocida* isolation (80%), followed by the emergency slaughtered calves, diseased calves and apparently healthy ones (42.9%, 32.7% and 20% respectively) as shown in Table 1.

Table 2 revealed the comparison between MAT and IHAT using somatic and capsular antigens respectively. Using MAT at the dilution 1/1280 the numbers of positive sera samples in the apparently healthy calves were 5 samples (10%), and 85 (54.5%) among the diseased ones. The number of positive sera

Table 2 Comparison between MAT and IHAT for detection of *P. multocida* infection among serum samples collected from the apparently healthy, diseased and emergency slaughtered calves.

Dilution of serum samples	Results of MAT(using somatic antigen)						Results of IHAT(using capsular antigen)					
	Apparently healthy calves		Diseased calves		Emergency slaughtered		Apparently healthy calves		Diseased calves		Emergency slaughtered	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
1/10	0/50	0	0/156	0	0/15	0	0/50	0	1/156	0.64	0/15	0
1/20	1/50	2	2/156	1.28	4/15	26.67	4/50	8	0/156	0	1/15	6.67
1/40	1/50	2	8/156	5.13	0/15	0	1/50	2	4/156	2.56	0/15	0
1/80	3/50	6	1/156	0.64	3/15	20	2/50	4	5/156	3.21	0/15	0
1/160	1/50	2	1/156	0.64	1/15	6.67	3/50	6	15/156	9.62	1/15	6.67
1/320	4/50	8	2/156	1.28	2/15	13.33	2/50	4	0/156	0	2/15	13.32
1/640	20/50	40	2/156	1.28	0/15	0	13/50	26	0/156	0	1/15	6.67
1/1280	5/50	10	85/156	54.49	0/15	0	20/50	40	95/156	60.89	9/15	60
Total positive No.	35	70	101	64.74	10	66.67	45	90	120	76.92	14	93.33

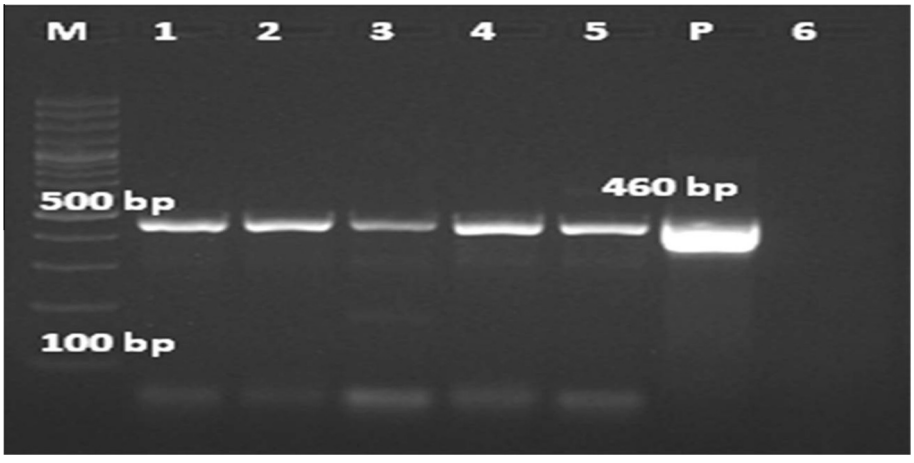

Figure 1 Agarose gel electrophoresis showing PCR amplified products of *P. multocida* field isolates. Lane M: PCR marker, Lanes 1–5: field isolates, Lane P: *P. multocida* reference strain ATCC No. 43137™ served as positive control and Lane 6: water served as negative control.

Table 3 Comparison between isolation, MAT, IHAT and ELISA for detection of *P. multocida* infection.

Source of the samples	No. of the samples	Isolation		No. of the samples	MAT (somatic antigen)		IHAT (capsular antigen)		ELISA (somatic antigen)		ELISA (capsular antigen)	
		No. of positive	%		No. of positive	%	No. of positive	%	No. of positive	%	No. of positive	%
Apparently healthy calves	50	10	20	50	35	70	45	90	10	20	21	42
*					5	10	20	40				
Diseased calves	156	51	32.7	156	101	64.74	120	76.92	56	35.9	145	92.9
*					85	54.49	95	60.89				
Emergency slaughtered calves	35	15	42.9	15**	10	66.67	14	93.33	7	46.7	12	80
*					0	0	9	60				
Total	241	76	31.5	221	146	66.1	179	80.9	73	33.03	178	80.5

* At dilution 1/1280.
** Positive for isolation of *P. multocida*.

in emergency slaughtered calves was 2 (13.33%) at dilution 1/320. Using IHAT at dilution 1/1280, the number of positive sera in the apparently healthy calves was 20 (40%), 95 (60.89%) in diseased calves and 9 (60%) in emergency slaughtered ones.

The number of positive sera for *P. multocida* infection by ELISA using somatic antigen was 73 (33.03%), while it was 178 (80.5%) using capsular antigen (Fig. 1 and Table 3).

4. Discussion

P. multocida causes haemorrhagic septicaemia (HS), which is a severe epidemic disease in cattle and buffaloes (Worarach et al., 2014). The present study, revealed the occurrence of *P. multocida* in the examined calves. The isolated *P. multocida* was positive for oxidase, catalase, indole and ornithine decarboxylase whereas, negative for gelatin liquefaction, did not hydrolyse urea and did not ferment mannitol and lactose (McFadden et al., 2011). API 20E system is useful in applying most of the biochemical tests and it was developed in the 1980s (Groom et al., 1986), but there appears to be no evidence of their routine use for the identification of *P. multocida*. The complexities associated with conventional diagnostic methods for *P. multocida* can be overcome by PCR. The PCR assay developed by Townsend et al. (1998) based on KMT1T7 and KMT1SP6 primers has been widely employed worldwide for initial species identification with field isolates irrespective of capsular types. All isolated of *P. multocida* produced a positive signal by an amplicon of approximately 460 bp.

A total of 88 *P. multocida* strains were isolated from the collected samples (256). Worarach et al. (2014) characterized 87 haemorrhagic septicaemia isolates caused by *P. multocida* from cattle and buffaloes, the majority of the isolates (88.5%) belonged to serotype B: 2, while 11.5% shared with serotype B: 2, 5. The incidence of *P. multocida* was 29.6% in the nasopharyngeal swabs of the apparently healthy (20%) and diseased calves (32.7%). While it was 54% among lung samples collected from dead (80%) and emergency slaughtered calves (42.9%). Naz et al. (2012) isolated *P. multocida* from diseased buffaloes in a percentage of 80% as shown in Table 1, while Hotchkiss et al. (2010) recorded that the prevalence of *P. multocida* was 17% in Scottish calves. In the present study all *P. multocida* isolated from apparently healthy, diseased and dead calves caused death to inoculated mice within 24–36 h post inoculation. These results revealed the high virulence of *P. multocida* (Naz et al., 2012). This is in accordance with the fact that healthy animals have been known to harbour virulent *P. multocida* and the apparently healthy animals could be the source of sporadic outbreak.

A better target for *P. multocida* detection would be a homogeneous antigen that could be easily purified and that is present in all isolates. The present study aimed to diagnose HS using MAT, IHAT and ELISA. The agglutination test was performed in twofold serial dilutions of serum. Sera with a titre of 1:16 were considered as positive Prado et al. (2006).

Antibody titre was detected in the examined apparently healthy calves. Among six apparently healthy cattle recognized by Haji Hajikolaei et al. (2008) as carriers of *P. multocida*, 5 animals were positive serologically and 2, 2, and one of them had titres 1:128, 1:64, and 1:32, respectively. It may be contaminating the environment. When the resistance of a herd is lowered, susceptible animals may become infected with a virulent strain present in a resistant host. Healthy carriers are common to all *P. multocida* infections and play a significant role in the epidemiology of these infections. Zaoutis et al. (1991) confirmed that testing for serum antibodies against the *P. multocida* is a reliable diagnostic tool to screen colonies for *P. multocida*.

The comparison between MAT and IHAT as serodiagnostic test for *P. multocida* revealed that the frequency of positive sera was higher when using IHAT than using MAT in apparently healthy calves, diseased calves and emergency slaughtered ones as shown in Table 2. Dziva et al. (2008) successfully detected *P. multocida* in the sera of calves suggesting a potential use of IHAT and MAT in diagnosis. Kawamoto et al. (1994) investigated a serological survey of haemorrhagic septicaemia in American bison and showed that IHAT was the most practical test among passive immunity test. Dziva et al. (2008) demonstrated antibodies against *P. multocida* capsular serotypes B and E in calves by IHAT. The IHA titres of *P. multocida* antibodies were detected in 212 (84.8%) of 250 cattle tested by Haji Hajikolaei et al. (2008). These results revealed that capsular antigen is more sensitive than somatic antigen.

ELISA test revealed that capsular antigen is more suitable to be used in ELISA. Many researchers used differently prepared antigens to be used in the diagnosis of *P. multocida*, Marshall et al. (1981) used sonicated whole cells of *P. multocida* as an antigen source, Dawkins et al. (1990) identified HS-causing organisms by ELISA using a live or formalin inactivated suspension of *P. multocida*, Prado et al. (2006) used outer membrane protein-based ELISA. Our results revealed that ELISA is more sensitive to diagnose *P. multocida* in apparently healthy, diseased and emergency slaughtered animals with percentages of 42%; 92.9% and 80% respectively as shown in Table 3.

In our study, using somatic antigen, 5 serum samples for MAT and 8 for ELISA in culture-positive samples of the emergency slaughtered calves were serologically negative. Also using capsular antigen 1 serum sample for IHAT and 3 for ELISA in culture-positive samples of the emergency slaughtered calves were serologically negative. Therefore, antibody detection coupled with the isolation of bacteria is recommended for the diagnosis of pasteurellosis. It may depend on the differences between immune responses to capsular types. Capsular types B and E elicited a strong immune response (Kawamoto et al., 1994). Therefore, the use of ELISA in serodiagnosis of capsular type infection may be recommended.

The data revealed that MAT, IHAT and ELISA antibodies could be detected from cultured negative samples collected from nasopharyngeal swabs of diseased calves. The antibody was detected by Kawamoto et al. (1994) from 22 (22%) of 100 samples of nasal culture negative rabbits. In seropositive animals of which the organism could not be isolated from nasopharynx, the organism might persist in other organs like tonsil and the animals were latent carriers (Haji Hajikolaei et al., 2008).

Kristensen et al. (2004) mentioned that ELISA could be used as a diagnostic tool for haemorrhagic septicaemia in endemic countries. Kawamoto et al. (1994) showed that ELISA was superior in serodiagnosis of rabbit pasteurellosis to IHAT and GDPT. The sensitivity of the serological test is dependent on the *P. multocida* antigen used Kawamoto et al. (1994).

5. Conclusion

It could be concluded that *P. multocida* could be incriminated as a cause of mortality in calves as they were purely isolated from the internal organs from necropsied animals. MAT and IHAT are serodiagnostic tests that are more diagnostic for *P. multocida* when compared with isolation and identification; this may be attributed to the misuse of antibiotic in farms before collection of samples. ELISA test was more sensitive in indicating reactor (positive) animals than the culture method, MAT or the IHA, as significantly low number of animals that were tested was found to be negative. The capsular antigen of *P. multocida* could be considered a potential diagnostic antigen.

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