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Short Communication

Isolation and Identification of *Pasteurella* multocida by PCR from sheep and goats in Fars province, Iran

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ABSTRACT

During one year period from 2010 to 2011 the samples from pneumonic animals were taken and transported to the laboratory. *Pasteurella multocida* were identified in 16.6% of animal by biochemical test. The high incidence of *P. multocida* was obtained in the south of Fars province, where the area was warm region. The Mean Death Time between the isolates was 12-18 and 19-24 hours. Only the capsular type A was identified in all the isolates and it is agreement with the finding by others, they indicated type A is the dominant type of *Pasteurella multocia* in tropical and sub tropical climate.

Keywords: *Pasteurella*, PCR, Toxigenic, Type, Iran

INTRODUCTION

Pasteurella (P) multocida causing pneumonic and septicaemic pasteurellosis in sheep, goats, and humans (Alwis 1996). It has been implicated in a number of diseases that manifest differently in various hosts and is also restricted to a specific geographical region (Blackall & Miflin 2000). According to polysaccharide capsules on their surface, they were differentiated into five serogroups including A, B, D, E and F (Chung *et al* 1998). Serogroup A and D of *P. multocida* have been incriminated agents of pneumonic Pasteurellosis in sheep and goats (Chandrasekaran *et al* 1991, Zamrisaad *et al* 1996). These isolates synthesize a 145-kDa toxin encoded by the chromosomal *toxA* gene. The *toxA* protein is an essential virulence factor for progression of Pasteurellosis in sheep and goats; in particular those of capsular serotypes A and D. Non-toxigenic *P. multocida* isolates do not cause the disease (Lichtensteiger *et al* 1996). Toxigenic *P. multocida* from an infected herd is distributed to clean herds by asymptomatic carriers, however many epidemiological and ecological aspects of disease and the pathogen remain unknown. *P. multocida* is part of normal flora of the upper respiratory tract, therefore predisposing factors such as overcrowding and bad ventilation, causes pasteurellosis (Mustafa *et al* 1978, Shayegh *et al* 2008, Hawari *et al* 2008). *P. multocida* is an endemic disease in Iran such as West Azarbaijan, Mazandaran, Gilan, Khozestan (Tabatabaei *et al* 2002, Shayegh *et al* 2009). But no information about the disease and prevalence in Fars province is available. There are several methods for

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isolation and identification of *Pasteurella sp*. They include bacterial culture, mice bio assay and antiserum base methods (Carter 1955, Heddleston *et al* 1972), meanwhile some of these such as culturing bacterial isolation are time consuming and give false negative results. Mice inoculation is not practical for identification (de Jong 1992). Therefore, the purpose of this study was to isolation and identification of *Pasteurella sp* by routine and molecular methods from sheep and goats in Shiraz Iran.

MATERIALS AND METHODS

Sample. One hundred and twenty nasals and tonsil swab samples were collected from ailing sheep and goats during 2010 to 2011 in cold and warm region of Fars province, Iran. Lung and liver lymph node, and tissue samples of the spleen, liver and lungs were collected from dead animals. The samples were then transported to the laboratory in cold condition. Biochemical tests were carried out on all 120 sample isolates using Entero rapid 24 test kits as described by Tefera (2002).

Mice assay. Twenty isolates identified as *Pasteurella* strains by biochemical test were inoculated separately in brain heart infusion (BHI) broth and incubated in a shaking incubator (100 rpm) at 37 °C overnight. Twenty groups of balb/C mice (3 mice in each group) were injected (0.2ml) via the intra peritoneum method. Three mice were left as control group and injected with only fresh BHI broth. Injected mice were observed for 24 hours for the fatality rates. The impression smears of the spleen, liver and lung were collected from the dead mice. Liver and lung samples from the dead mice were streaked onto sheep blood agar and incubated as the same above.

DNA extraction. DNA extraction was carried out according to described by Ozbey *et al* (2004) with minor modifications. Briefly, overnight BHI culture was centrifuged. The pellets were washed twice in PBS. The washed pallets were resuspended in trice EDTA **(**ethylenediaminetetraacetate) (pH, 7.3). The cell was extracted with phenol previously saturated with trice EDTA (pH, 7.3) and centrifuged. The upper phase centrifuged solution was collected and transferred to clean micro tube. The content was mixed with phenol/chloroform/isoamyl and centrifuged as well. After addition sodium acetate and absolute ethanol, DNA was precipitated. DNA was dried and resuspended in EDTA and reserved in freezer until used. Identification of *P. multocida* was confirmed by PCR using primers set in table 1. The *kmt1* gene was amplified by *all pass* primer. This gene indicated the species of *P. multocida* among the isolates. The PCR reaction was performed in a total volume 25 µL containing 0.4 µM from each primer, 0.2 mM dNTPs, 1X Taq reaction buffer, 2.5 mM $MgCl_2$, 1.25 U from Taq DNA polymerase and1 µL from the isolated DNA (all reagents made from Sina-gene, Iran). The DNA was amplified under the following conditions in a thermo cycler (gradient master cycler Eppendorf, Germany) include: primary denaturation step at 94 °C for 4 min, 35 cycles using the following settings: initial denaturation at 94 °C for 45 sec, annealing at 45 °C for 45 sec and extension at 72°C for 45 sec, followed by 5 min at 72 °C. The PCR product was analyzed on 1.3% agarose gel stained with ethidium bromide. Two DNA ladders 1 kb and 100 bp (Fermentas) were used to determine the size of the amplified fragments. The results were then analyzed under SPSS 12.5 with ANOVA software.

RESULTS AND DISCUSSION

The isolates subjected to biochemical tests were positive for indole, nitrate reduction, oxidase and catalase and negative for MR VP and simmons citrate test. The isolates fermented glucose, fructose, mannitol, trehalose and sucrose. According to the biochemical results, twenty (16.6%) of the 120 samples were identified as *Pasteurella*. On the basis of these results, *P. multocida* are the most common in sheep and goats in the warm region of Fars province (P<0.50%).

Table 1. Sequences of the oligonucleotides used for detection of *P. mutocida*

Primer	Gene	Name	Sequence	Amplimer size(bp)
All Pass	*KMT1*	KMT1T7	ATCCGCGATTTACCCAGTGG	460
		KMTSP6	GCTGTAAACGAACTCGCCAC	
Capsular type A	*hydD-hydC*	CAPA-FWD	TGCCAAAATCGCAGTCAG	1044
		CAPA-REW	TTGCCATCATTGTCAGTG	
Capsular type D	*dcbF*	CAPD-FWD	TTACAAAAGAAAGACTAGGAGCCC	760
		CAPD-REW	CATCTACCCACTCAACCATATCAG	
Dermo necrotic toxin	*toxA*	TOXA-FWD	TACTCAATTAGAAAAAGCGCTTTATCTTCC	300
		TOXA-REV	TCCCAGTAATTTGTCTGTATTTTATCAAAT	

Table 2. Biochemical, mice assay and PCR results of isolates from sheep and goats

Isolates	**Biochemical test**	**Dead mice**	**P. m (*kmt1*)**	**Type A Cap(*hydD-hydC*)**	**DNT (*toxA*)**	**Type D Cap(*dcbF*)**
120	20(16.6%)	16(13.3%)		10 (8.33)		-

Table 3. Capsular typing and *tox A* frequency of *P. multocida* isolates

Isolates	1	2	3	4	5	6	7	8	9	10
All pass	+	+	+	+	+	+	+	+	+	+
Capsular A	+	+	+	+	+	+	+	+	+	+
Capsular D	-	-	-	-	-	-	-	-	-	-
ox A	+	+	+	+	+	+	+	+	+	+

The serotypes of the isolates were determined due to PCR analysis. The serotypes of the isolates were determined due to PCR analysis.The 12 mice groups injected with isolates identified by biochemical test were found to be virulent and died within Mean Death Time (MDT) between 12-18 hrs and four isolates had a low virulence with MDT between 19 and 24 hours. Whereas, all three mice injected with BHI broth culture as control did not die even after two days post injection (P<0.05%) (Table 2). Liver and spleen impression smear evidenced characteristic of *Pasteurella* on gram staining. The isolates showed typical morphological and cultural characteristics of dew drop, mucoid, non haemolytic colonies in blood agar. No growth was observed in MacConkey agar. Culture smears revealed characteristic gram negative coccobacillary organisms. PCR assay for *P. multocida* species specific developed by Townsend et al. (1998) with some modification. The method identifies the subspecies of *P. multocida* by amplifying 460 bp DNA fragment within KMTI gene using the primers KMTISP6 and KMTIT7. This indicated whether the species is *P. multocida* or not (figure 1). Table 3 was shown the results of multiplex PCR (mPCR) based on primer set as in table 1. According to amplification of hya C – hyaD gene of *P. multocida* by PCR, all the isolates were identified as capsular type 'A' and the amplicon size was found to be 1044bp (figure 1). *P. multocida* has also been reported as a cause of pneumonic pasteurellosis in sheep and goats (Jaglic *et al* 2005, Xibiao *et al* 2009).

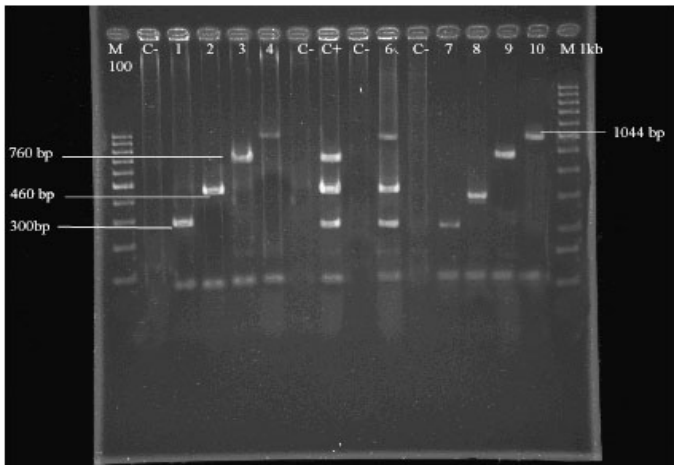

Figure 1. 1.4% agarose gel electrophoresis for specific species (*kmt1*), capsule (*capA*) and toxigenic (*toxA*) primers for Pasteurella. Lane M: 100 bp and 1 kb ladder, line 1-10: virulent factors of *P. multocida* isolates, C+: positive and C-: negative controls.

Sheep and goats pneumonia is especially common in newborn and in feedlot lambs, it can also occurs in the

mature ewe flock with milder clinical signs. Pneumonia occurs in all ages of sheep and goats, in all breeds, in every country of the world (Ozbey *et al* 2004). The high incidence of disease in warm region, exhibition a positive correlation with area, suggesting the climate condition plays a role in respiratory problems in that area. Factors such as crowding, dust, damp humid weather, or stress all can increase the disease (Weiser *et al* 2003). The mouse bio assay findings were comparable with the findings of Mustafa *et al* (1978) and Diallo *et al* (1995). These results were also in accordance with observation of Holmes (1998) and OIE manual (2004). They were reported 50% of the isolates with minimum MDT. The finding of biochemical results are in agreement with Tefera (2002) and OIE manual (2004). They found 17.5% according to biochemical test. The *P. multocida* isolates that were positive by biochemical reactions subjected also for possible positive results by PCR. No amplifications were produced from the negative control. These results were in agreement with Ozbey and Muz (2006), who mentioned that some *P. multocida* isolates that were positive by culture were also detected to be positive by PCR. Methods like biochemical tests using API 20E, and mice bio assay were not detected any of the virulence factors. (McAvin *et al* 2001). Oligonucleotide primers designed for these isolates have formed the basis for mPCR assays that specifically identify *P. multocida* and, in particular, capsular type and toxigenic species isolates that cause severe disease (Chandrasekaran *et al* 1991; Zamrisaad *et al* 1996). Similar results were not obtained with biochemical test and mice assay. It also indicated that molecular typing methods can provide a stable and highly useful analysis of bacterial isolates and have proved to be beneficial in reducing the limitations of the biochemical tests (Hawari 2008). The findings of this study are in agreement with reports that *P. multocida* type A are the most prevalent in Iran (Tehrani *et al* 2004, Shayegh *et al* 2008). Our findings support the need for the development of a vaccine using the most prevalent *P. multocida* serotypes as well as strategic deworming, and improved housing conditions for sheep and goats in the Fars province and totally in Iran.

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References

Alwis, D. E. (1996). Haemorrhagic septicaemia: Clinical and epidemiological features of the disease. International workshop on diagnosis and control of HS. Bali, Indonesia**,** 28-30.

Blackall, P. J. and Miflin, J. K. (2000). Identfication and typing of pasteurella multocida:a review. *Avian Pathology* 29**:** 271-287.

Carter, G.R. (1955). Studies on Pasteurella multocida: a haemagglutination test for the identification of serological types. *American Journal of Veterinary Research* 16**:** 481-484.

Chandrasekaran, S., Hizat, K. and Saad, Z. (1991). Evaluation of combined Pasteurella vaccines in control of sheep pneumonia. *British Veterinary Journal* 147**:** 437-443.

Chung, Y. J., Zhang, Y. and Adler, B. (1998). The capsule biosynthetic locus of Pasteurella multocida A1. *FEMS Microbiology Letters* 166: 289-296.

De Jong, M. F. (1992). (Progressive) atrophic rhinitis. In A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor (ed.). Diseases of swine, 7th ed**,** p. 414–435.

Diallo, I. S., Bensink, J. C., Frost, A.J. and Spradbrow, P.B. (1995). Molecular studies on avian strains of *P.multocida* in Australia. *Veterinary Microbiology* 46: 335-342.

Hawari**,** A. D., Hassawi**,** D. S. and Sweiss M. (2008). Isolation and Identification of *Mannheimia haemolytica* and *Pasteurella multocida* in Sheep and Goats using Biochemical Tests and Random Amplified Polymorphic DNA (RAPD) Analysis. *Journal of Biological Science* 7: 1251-1254.

Heddleston, K. L., Gallagher, J. E. and Rebers, P. A. (1972). Fowl cholera: gel diffusion precipitin test for serotyping Pasteurella multocida from avian species. *Avian Diseases* 16**:** 925-936.

Holmes, B. (1998). *Pasteurella In:* Topley and Wilson's microbiology and microbial infections. 9th Edn. Balow, A

and B.I. Overden, Systematic bacteriology. 2nd Volume, Oxford University Press, New York.

Jaglic, Z., Kucerova, Z. and Nedbalcover, K. (2005). Characterization and comparison of Pasteurella multocida isolated from different species in the Czech Republic : Capsular PCR typing, ribotyping and dermonecrotoxin production. *Veterinary Medicine Czech* 50: 345-354.

Lichtensteiger, C.A., Steenbergen, S. M. and Lee, R. M. (1996). Direct PCR Analysis for Toxigenic Pasteurella multocida. *Journal of Clinical Microbiology* 34**:** 3035–3039.

McAvin, J. C., Reilly, P. A. and Roudabush, R. M. (2001). Sensitive and Specific Method for Rapid Identification of Streptococcus pneumoniae Using Real-Time Fluorescence PCR. *Journal of Clinical Microbiology* 39**:** 3446-3451.

Mustafa, A. A., Ghalib, H. W. and Shigidi, M. T. (1978). Carrier rate of *P.multocida* in a cattle herd associated with an outbreak of Haemorrhagic septicaemia in Sudan. *British Veterinary Journal* 134: 375- 380.

Office International des epizooties. (2004). Manuals of standards for diagnostic test and vaccine. 4thedition, France, pp 446 – 456, 740 – 746, 921 – 926.

Ozbey, G. and Muz, A. (2006). Isolation of aerobic bacteria from the lungs of chickens showing respiratory disorders and confirmation of *Pasteurella multocida* by polymerase chain reaction (PCR). *Veterinarski Arhiv* 76: 217-225.

Shayegh, J., Atashpaz, S. and Hejazi, M. S. (2008). Virulence genes profile and typing of ovine Pasteurella multocida. *Asian Journal of Animal and Veterinary Advances* 3**:** 206-213.

Shayegh, J., Sharaf, D. J., Mikaili, P. and Namvar, H. (2009). Pheno- and genotyping of *Pasteurella multocida* isolated from goat in Iran. *African Journal of Biotechnology* 8: 3707-3710.

Tabatabaei, M, Liu, Z., Finucane, A., Parton, R and Coote, J. (2002). Protective immunity conferred by attenuated *aroA* derivative of *Pasteurella multocida* B:2 strains in a mouse model of hemorrhagic septicemia. *Infectious and Immunity* 70: 3355-3362.

Tefera, G. and Smola, J. (2002). The utility of Enterorapid 24 Kit for the identification of P. multocida *Veterinary Medicine Czech* 47**:** 99-103.

Tehrani, A. A., Ras, M. B. and Niazy, H. (2004). Isolation and identification of P. haemolytica biotype A from sheep in Urmia, Iran. *Iranian Journal of Veterinary Research, University of Shiraz* 5: 105-109.

Townsend, K.M., Boyce, J.D. and Chung, J.Y. (2001). Genetic organization of Pasteurella multocida cap loci and development of multiplex capsular PCR typing system. *Journal Clinical Microbiology* 39: 924-929.

Weiser, G. C., DeLong, W. J. and Paz, J. L. (2003). Characterization of Pasteurella multocida associated with pneumonia in bighorn sheep. *Journal of Wildlife Diseases* 39**:** 536–544.

Xibiao, T., Zhanqin, Z. and Junyong, H. (2009). Isolation, Antimicrobial Resistance, and Virulence Genes of Pasteurella multocida Strains from Swine in China. *Journal of Clinical Microbiology* 47**:** 951-958.

Zamrisaad, M., Effendy, A. W. M., Maswati, M. A. (1996). The goet as a model for studies of pneumonic pasteurellosis caused by Pasteurella multocida. *British Veterinary Journal* 152**:** 453-458.