Archives of Razi Institute, Vol. 69, No. 1, June (2014) 47-55

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DOI: 10.7508/ari.2014.01.007

Full Article

Isolation and Identification of *Pasteurella multocida* from Sheep & Goat in Iran

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Received 19 Nov 2011; accepted 27 Oct 2013

ABSTRACT

This study has been carried out with the objective of isolation and identification of agent(s) of pasteurella pneumonia in sheep and goat in Iran using bacteriological and biochemical assays to be identified in the pursuant researches to be used in pasteurellosis vaccine production. To accomplish this objective, samples were gathered from areas suspicious to pasteurellosis infection and industrial abbatoirs according to clinical and autopsy symptoms from eight provinces of Bushehr, Esfahan, Kerman, Kohgilooyeh & Boyr Ahmad, Fars, Qom, Tehran and Qazvin in a period from spring 2008 to spring 2011. Samples were different in sort due to the existent condition but generally were comprised of palatine tonsil swabs or blood samples taken from jugular vein in live animals and lungs or upper respiratory tract lymph glands in dead or slaughtered animals. Totally, 1454 samples (1120 samples of sheep, 334 samples of goat composed if 1084 samples of live animals and 370 samples of dead or slaughterd animals) were tested. Considering results obtained from assays, only 54 samples (3.71%) were assessed as being pasteurella, genus of which was totally identified as multocida.

Keywords: Isolation, Identification, Pasteurella multocida

INTRODUCTION

Ovine and caprine pneumonia is a syndrome originated by interaction between environmental stress factors, mico-organisms and host defense capability. The disease is seen in all breeds of sheep and goats, in all ages and all countries. It afflicts severe economic losses as well as heavy mortality in the flocks. The mortality rate of the disease in young lambs may reach upto 90 percent (Bobb 1999, Mohamed & Abdelsalam 2008). In this basis, a great work has been carried out to help control and prevent its occurrence in different countries. These struggles have been mainly concentrated on vaccine development. In outbreaks of acute ovine and caprine pneumonia, pasteurella especially *Mannheimia (Pasteurella) haemolytica* (which is the natural flora of upper respiratory tract of healthy animals) and *Pasteurella multocida* have been isolated more than other pathogenic agents from affected lungs. About 30% of domestic animal mortality is known to be related to pasteurella infection (Bobb 1999, Diker *et al* 2000). Mycoplasma,

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parainflenza virus type 3 arginini and *Mycoplasma ovipneumonia* can be mentioned as other pneumonia causing factors in sheep and goats (Goodwin-Ray 2006). A fever, cough, nasal and eye discharge as well as diarrhea is seen in acute pasteurellosis. Lesions reported in acute cases are haemorrhagic broncopneumonia accompanied by pleurisy and pericarditis. Lung lesions are limited to posterior and ventral areas (Hassani Tabatabaiee *et al* 2001). The causative agent is an aerobic, gram negetive, immobile and morphologically rod or in coccobacillus form (Mahmoud 2007, Thomas 1981). *Pasteurella multocida* is a gram negative, coccobacillus or rod and bipolar agent. It is an uncompulsary anaerobic bacterium, incapable to induce haemolysis, consisting of light gray colonies with a smooth surface and ridges, having a diameter of 1-2 mm (after 24 hours of heating in blood agar or agar chocolate) and a specific odor. The bacterium doesn't grow in MacConkey medium (Mahmoud 2007, Thomas 1981). The key biochemical tests to diagnose the bacterium are indol, oxidase, catalase and positive sensitivity to penicillin, but it is preferrable to use classic and commercial tests simultaneously. The classic tests include oxidase, catalase, indol, nitrate, motility, urease, ornithine, lysine, arginine, citrate, esculin, TSI and H_2S (Mahmoud 2007, Thomas 1981). One of the main problems in controlling ovine and caprine pasteurella pneumonia is lack of competence of developped vaccines which leads to ineffective and imperfect immunity. Although different kinds of live and killed vaccines have been produced to prevent the outbreaks of the disease throughout the world, but their efficacy has shown to be different on farms and sometimes, they have not only been ineffective but also have increased morbidity and mortality in the farm. Based on the surveys, considerable difference in immunogenicity of different pasteurella serotypes and limited protection against heterologue serotypes have been assessed as the reason of inefficiency of vaccine in inducing an efficient protection, so a constant surveillance of current serotypes of the area as well as controlling the antigenicity and immunogenicity of new serotypes to control pasteurella pneumonia and vaccine development seems to be necessary (Diker, Akan & Kaya 2000). This study has been carried out with the objective of isolation and identification of agent(s) of pasteurella pneumonia in sheep and goat using bacteriological and biochemical assays to get required species for performing complementary researches resulting in vaccinal species to manufacture sheep and goat pasteurellosis vaccines.

MATERIALS AND METHODS

Mat Collecting samples. Considering the existent reports of pasturella pneumonia outbreaks from different areas of Iran, eight provinces showing the greatest amount of morbidity including Bushehr, Esfahan, Kerman, Kohgilooyeh & Boyr Ahmad, Fars, Qom, Tehran and Qazvin were selected to gather the samples. So, in a period between spring 2008 to 2011, we referred to the farms which have reported the outbreaks of acute pasturella pneumonia and getting samples from animals showing fever, cough nasal and eye discharge and diarrhea (which, based on the existent data had received no medication and vaccination against pasteurellosis). The samples were different according to the conditions but in general, were comprised of palatine tonsil swabs or blood sample from jugular vein (2ml) in live animals and samples gathered from lungs (anterior and ventral lobes) or upper respiratory tract lymph glands (through aseptic autopsy on farm) from dead animals passing no more than 2 hours post-mortem. Furthermore, in each province, we were referring to the industerial abattoirs and taking swab or blood samples from animals showing symptoms of acute pasteurella pneumonia in pre-slaughter stage as well as gathering samples from lungs showing haemorrhagic bronchopneumonia accompanied by pleurisy especially in anterior and ventral segments together with pricarditis. Samples from lymph glands of upper respiratory tract were also taken.

Samples transportation. Swap samples were transported to the diagnostic laboratory in Stuart

Transport Medium (manufactured by HIMEDIA company) which contained 6µg/ml Vancomycine, 12.5 µg/ml Nystatine (to provide a relatively specific transport medium for pasteurella) and blood samples were transported in Brain Heart Broth (with the same specifications) along with ice cubes lasting not more than 72 hours.

Bacteriological assays. Under aseptic conditions in the lab, the delivered swabs, blood or tissue samlpes were cultured in blood agar containing %5 defibrinated sheep blood. 40 gr/l of blood agar base (manufactured by MERCK company), 6µg/ml of Vancomycine and 12.5µg/ml of Nystatine. These cultures were incubated for 24 hours in 37 ºC, then regarding the variety of grown bacteria on the medium, a re-culture on blood agar and purification was made after precisely checking size, color and forms of pasteurella colonies. If the result of growth in the mentioned medium, morphological studies (apparent forms of colonies on blood agar realizing the induction of haemolysis) and also the result for microscopic study of gram-stained smears were all positive, then the growth of bacterium on fluid medium of Brain Heart Broth (with the mentioned specifications) were being brought into account. In cases where culture of bacterium on fluid medium was also successful, catalase and oxidase assays were being done, and in cases where the sample was haemolysis-positive, the culture was made on MacConkey agar. In case of accordance of the mentioned test results to that of Pasteurella Standard, penicillin sensitivity test was being performed. In this assay, the bacterium was cultured in Brain Heart Broth medium and kept for 6 hours in37 ºC incubator. Then a semi-MacFarlane dilution was being prepared from the 6 hours incubated bacterium and was mass-cultured by a sterile swab on blood agar medium laying a penicillin disc (manufactured by HIMEDIA company) on the center of the medium. Finally, the medium was being kept in a 37 ºC incubator for 24 hours and then the result was being observed. Totally 1454 samples were delivered and tested. Number of samples separated by under-investigation provinces, sort of animal, health situation of the animal and sort of sample have been reflected in table 1.

Biochemical assays. In case, the sensitivity of isolate to penicillin was confirmed, and for getting insurance that the obtained bacterium is really pasteurella, the bacterium was cultured in differential medium of Kligler agar, ornithin decarboxylase broth, urease, MR-VP broth, SIM medium. In case of accordance of the obtained results to that of the pasteurella biochemical demonstrations, and with the aim of studying the isolate's pathogenicity and estimation of its virulence, the 6 hours long culture of the bacterium was made on Brain heart broth medium as stated before and 1 ml of it was injected intraperitoneally to two 20-24 gram weighted mice and the result was monitored for 24 hours. When the mice died, we got blood from their heart and lung tissues under aseptic conditions making a direct culture on blood agar, the isolation and identification was carried out again according to the mentioned procedures. To complete the identification process and acquiring a tentative diagnosis of genus and species and providing required conditions for identifying biotypes of isolates through carbohyderates fermentation, we used differential diagnosis kits from HIMEDIA Company.

Storage of Bacteria. In case, the obtained results were in accordance to that of the pasteurella biochemical table, the isolates were being kept for the future proceeding. To accomplish this, the 6 hours culture of bacterium on Brain heart broth was prepared, then mass-cultured on blood agar being kept for 12 hours in a 37 ºC incubator. Finally, the colonies were rinsed by skimmed milk adding 0.2 ml of it to Edward Tube and frozen using a lyophilizer located in the Department and was stored on -20 ºC.

RESULTS

Out of 1454 gathered samples, 49 samples were eliminated due to absence of growth or growing colonies perfectly different with Pasteurella in the primary culture. Of the remaining samples, 1002 were eliminated in purification and morphological study

phase and 45 were eliminated due to absence of growth in liquid medium. Realizing that this research has been oriented to attain required species for complementary studies ending to vaccinal species to develop sheep and goat pasteurellosis vaccine, studying the growth ability of isolates in liquid media was of great importance. Focusing on the results acquired from catalase and oxidase tests as two indicator tests in diagnosis of pasteurella, resulted in elimination of 112 more samples. Growing some of these samples in MacConkey regarding non-resemblance of grown colonies to that of growable species of pasteurella in the called medium (such as *Mannheimia haemolytica*) also absence of sensitivity to penicillin in some of samples, realizing that this test is indicator in diagnosis of pasteurella genus, reduced assessable isolates to 182 samples. Performing biochemical tests regarding some of them such as Motility, Indol, Urease, MR-VP and Glucose Fermentation, caused uncertainty for 7 isolates about being real pasteurella. With the aim of pathogenicity study and acuity estimation, the remaining 175 isolates were injected to mice. Out of them, only 90 samples caused mortality in the animals. Replication of all of these tests on samples of died mice confirmed that 54 isolates were pasteurella. The obtained results were confirmed using differential diagnosis kit produced by HIMEDIA, and comparing them to pasteurella standards, 54 isolates were finalized. The obtained results of these tests have been detailed in table 2.

DISCUSSION

Based on the data given on table 1, out of total 1454 delivered samples, 1120 samples (%70.02) belongs to sheep and 334 samlpes (%29.98) to goat whereas regarding content of table 3, %3.21 of isolates belongs to sheep and %5.39 to goat. This fact proves that the probability of isolation of disease agent in goat is more than that of sheep. According to the data presented in table 3, the maximum percentage belongs to nasal swabs (%5) and in lower rank; it belongs to tonsil swabs (%2.62), blood (%2.52) and lung tissue (%1.73). No bacteria were isolated from lymphatic gland tissue samples. The result does not accord to that of research carried out by Dunbar et al. (1990) and Wild & Miller (1991), but it is near to the results obtained by Deressa et al. (2010). Totally 1454 samples were studied in this research. Most of the samples were individual and some were compositive, this means that various gathered samples from a unique animal were transported altogether in a transport medium to the laboratory and hence were cultured on a culture medium sharedly. Based on the data recorded on table 3, only 1405 samples out of 1454 achieved primary growth on blood agar. Defeat in primary growth can merely be due to the exposition of transport medium in unfavorable conditions or elongation of transport time towards laboratory. Out of 1405 grown samples in primary culture medium, only 403 cases could be purified. The reason for this fact was high bacterial variety in primary culture medium and lack of competence of pasteurella against other bacteria. Considering the objective of this research which was infact isolation and identification of pasteurella pneumonia agent(s) in sheep and goat to procure required biotypes for accomplishment of complementary research resulting in vaccinal biotype to be used in production of sheep and goat pasteurellosis vaccine, as well considering the pasteurellosis vaccine production process which is based on bacterium growth in liquid medium, a survey on possibility of growing isolates in liquid medium was done and resulted in elimination of 45 samples due to lack of growth potency in the mentioned medium (despite repeated culture). Lack of growth potency of the bacterium in liquid medium can merely be attributed to the individual characteristics of bacterium. Considering the results of catalase and oxidase assays also regarding that these two assays act as indicator index (based on researches carried out by Thomas 1981, Kirkan *et al* 2005, Ilhan *et al* 2007, Hawari *et al* 2008 and Prabhakar *et al* 2010), merely 246 samples were conveyed to the next step. Because of the unique characteristics of pasteurella in growth/lack of growth in MacConkey medium, 246 samples were cultured in

Table 1. List of delivered samples from under-investigation provinces based on animal, its health situation and sort of sample

Province	Number of delivered samples	Sort of the animal		Animal's health situation		Sort of sample*				
		Sheep	Goat	Live	Dead	Nasal Swab	Tonsil Swab	Lung Tissue	Lumph Gland Tissue	Blood
Fars	700	549	151	550	150	517	462	150	149	118
Esfahan	285	225	60	166	119	162	3	103	16	1
Kerman	208	159	49	171	37	93	78	37	0	0
Qom	134	68	66	114	20	101	13	19	1	0
Tehran	70	70	0	40	30	40	0	28	2	0
Bushehr	30	30	0	26	4	13	13	2	2	0
Kohgilooyeh & Boyr Ahmad	14	6	8	8	6	5	3	3	3	0
Qazvin	13	13	0	9	4	9	0	4	0	0
Sum	1454	1120	334	1084	370	940	572	346	173	119

* The total sum of different delivered samples exceeds 1454 because; in some cases more than one sort of sample was gathered.

Table 2. Conclusion of the test results

No	Title of the test	Acceptable samples after testing
1	Primary culture and studying bacteria growth on blood agar	1405
2	Purification and morphological studies	403
3	Growth study in fluid medium and morphology	358
4	Catalase*	297
5	Oxidase*	285
6	Catalase & Oxidase*	246 (Number of acceptable samples regarding the results of both tests)
7	Growth on MacConkey	68
8	Morphological similiarity of grown colonies on MacConkey to that of pasteurella	9
9	Sensitivity to penicillin	182
10	Motility*	179
11	Indol*	179
12	Urease*	179
13	MR-VP*	179
14	Ornithin decarboxylase	179
15	Gas production monitoring	179
16	H_2S production monitoring	179
17	Lactose fermentation	179
18	Glucose fermentation*	175
19	Injection to laboratory animal	90
20	Isolation of bacteria from different organs of dead laboratory animal	54
21	Differential asseys (differential diagnosis kit manufactured by HIMEDIA including: ONPG; Lysine utilization; Ornithin utilization; Urase; Phnylalanine Deamination; Nitrate reduction; H2S production; Citrate utilization; Voges proskauers; Methyl red; Indol; Malonate utilization; Esculin hydrolysis; Oxidase; Fermentation of Arabinose; Xylose; Adonitol; Rhamnose; Cellobiose; Melibiose; Saccharose; Raffinose; Trehalose; Glocose and Lactose)	54

* Index asseys for decision making

Table 3. Number of isolates separated by province, health situation of the animal and sort of sample

Name of Province	No of isolates	Sort of animal		Health situation of the animal		* Sort of sample				
		Sheep	Goat	Live	Died	Nasal swab	Tonsil swab	Lung tissue sample	Lymphatic gland tissue sample	Blood
Fars	15	11	4	15	0	15	14	0	0	3
Esfahan	14	11	3	13	1	13	0	1	0	0
Kerman	2	2	0	2	0	1	1	0	0	0
Qom	18	7	11	13	5	13	0	5	0	0
Tehran	5	5	0	5	0	5	0	0	0	0
Bushehr	0	0	0	0	0	0	0	0	0	0
Kohgilooyeh & Boyr Ahmad	0	0	0	0	0	0	0	0	0	0
Qazvin	0	0	0	0	0	0	0	0	0	0
Total	54	36	18	48	6	47	15	6	0	3
Percentage of isolates	100	66.66	33.34	88.88	11.12	87.03	27.77	11.11	0	5.55
Percentage of total samples	3.71	3.21	53.39	4.43	1.62	5	2.62	1.73	0	2.52

* The total sum of different delivered samples exceeds 54 because; in some cases more than one sort of sample was gathered and cultured in a compiled form.

Table 4. Conclusion of test results on isolates

No	Title of the assey	Positive responses out of 54 isolates	Percentage of positive responses
1	haemolysis	0	0
2	catalase	54	100
3	oxidase	54	100
4	MacConkey agar growth	5	9.26
5	Colony similarity on MacConkey	0	0
6	Penicillin suseptibility	54	100
7	motility	0	0
8	indol	43	79.6
9	urease	0	0
10	MR	0	0
11	VP	0	0
12	Ornithine decarboxylase	23	42.6
13	Gas production	0	0
14	H2S production	1	1.85
15	ONPG	5	9.25
16	Lysine decarboylase	25	46.29
17	Nitrate reduction	53	98.14
18	Citrate utilization	0	0
19	Esculin hydrolysis	2	3.70
20	Phenylalanine deamination	0	0
21	Malonate utilization	0	0
22	Glucose	54	100
23	Lactose	1	1.85
24	Arabinose	21	38.88
25	Xylose	46	85.88
25	Adonitol	0	0
26	Rhamnose	0	0
27	Cellobiose	0	0
28	Melibiose	0	0
29	Saccharose	24	44.44
30	Raffinose	13	24.07
31	Trehalose	31	57.40

in this medium. 68 samples showed growth in MacConkey medium and 9 samples had morphological similarity (form of colony and its shape in staining) to that of pasteurella (red pin point colonies which is characteristics to *Pasteurella haemolytica*). Investigating sensitivity to penicillin was the next assay which was done for all the remaining samples. Regarding definite sensitivity of pasteurellas to penicillin, 64 samples out of 246 were eliminated due lack of sensitivity to penicillin. 3 samples out of 182 remainig samples were eliminated due to motility and 4 samples due to lack of potency in glucose fermentation. Regarding the aim of this research, studying pathogenicity of isolates was very important and this study was done based on researches carried out by Mahmoud (2007), Prabhakar et al. (2010) via injection of remaining isolates (175 samples) to lab animal (mouse). 90 isolated succeeded to induce the disease and mortality in lab animal during 24 hours after injection and 85 samples induced no lesions, so they were eliminated. As some of the species and biotypes of pasteurella lack pathogenicity and that the bacterium is a part of natural flora of sheep and goat upper respiratory tract, this result was not unexpected.

Out of all died animals, only 54 isolates were madeandidentified as pasteurella. Induction of mortality in lab animal through secondary infection due to contamination during injection, carcass putrification due to time elapse between death and time of gathering samples as well, induction of secondary infection on the time of getting samples from carcass, were all the reasons of inability in isolation and identification of pasteurella from all died animals.

Genus of the bacterium. Based on what was stated, especially the results obtained from morphology and catalase, oxidase, sensitivity to penicillin, motility, urease, MR-VP and glucose fermentation, merely 54 samples (%3.71) were identified as being pasteurella. The stated assays have also been emphasized as indicator index assays for pasteurella identification during researches carried out by Thomas (1981), Hawari et al. (2008) and Probhakar et al. (2010). Percentage of isolates per total samples (without pathogenicity investigation) has been reported by Tehrani et al. (2004) as %16 and Ezzi et al. (2007) as %2.31. Based on the table 4, the results obtained from asseys including haemolysis, catalase, oxidase, motility, indol, urease, MR ,VP, nitrate reduction, citrate utilization, phenylalanine deamination, malonate utilization, glucose, lactose, xylose, adonitol, rhamnose and cellobiose on 54 isolates have similiarity with that of Thomas (1981), Hawari et al. (2008) and Probhakar et al. (2010), but the results obtained from ornithine decarboxylase, H_2S production, ONPG, lysine decarboylase, esculin hydrolysis, arabinose, saccharose, raffinose and trehalose assays does not have any similarity to that of the mentioned studies. Species of the bacterium. Focusing on the results obtained from carried out assays, especially haemolysis, growth in MacConkey medium, indol and lactose fermentation, the species of isolates is multocida, which compared to the investigations carried out inland and what has been reported by Ezzi et al. (2007) is accordant, but it is in controversy with what has been reported by Sasani et al. (2002) and Tehrani et al. (2004). They isolated *Pasteurella haemolytica biotype A* from 317 samples (%16) out of 1988 sheep lung tissues in a bacteriological and pathological study during May 1998 to April 1999 in Urmia, Iran. As the methodology used by Tehrani et al. (2004) except the primary steps (gathering samples and dispatching them to lab before culture on blood agar medium) does not have a significant difference with that of this study, so the procedure cannot be calculated as an influencing factor on difference between the results, and merely, difference in studied areas and difference of time between the two studies (more than 10 years), as well, changes in disease inducing factors during the period can be realized as a reason for this important fact. Compared to the results obtained from similar studies in abroad, these results do not accord with that of Ayelet et al. (2004), Kirkan et al. (2005) and Ilhan & Keles (2007), but are in accordance with that of Mahmoud (2007), Hawari et al. (2008) and Prabhakar et al. (2010). The reason for this inaccordance to the

mentioned studies can merely be attributed to difference in disease agent according to studied areas.

Bacterial biotype. Regarding the results obtained from assays especially fermentation of carbohydrates (table 4) and their comparison to the similar researches including what has been reported by Thomas (1981), isolates include 4 biotypes of B, E, F and J with 29, 7, 2 and 6 isolates respectively. Shayegh et al. (2010) identified the biotypes of all their 9 ovine and caprine isolates except one case as A. Ayelet et al. (2004), Kirkan et al. (2005), Ilhan & Keles (2007) and Prabhakar et al. (2010) too, identified all or the majority of pasteurella isolates as being biotype A in their research. Based on this fact, reliance on the results of carbohydrates fermentation assay does not seem to have authenticity for assessment of pasteurella biotype.

Acknowledgment

This research was carried out by financial support of Razi Vaccine and Serum research Institute and co-operation of Bacterial Diseases Fighting Department of State Veterinary Oranization, Veterinary General Administrations of Bushehr, Esfahan, Kerman, Kohgilooyeh & Boyr Ahmad and Qom provinces, Fars Branch of Razi Vaccine and Serum research Institute, Esfahan Research Center for Agriculture and Natural Resources as well as Ray & Ziaran abattoirs. Many thanks for their kindness and feeling responsibility. The authors also dedicate their best thanks for Mrs. Dr. Sedigheh Kazeminia, Mr. Dr. Mansour Banani, Mr. Dr. Morad Moradi Garavand, Mr. Dr. Reza Mohammadi, Mr. Dr. Seyed Mohammad Barani, Mr. Dr. Mohammad Reza Heidari, Mr. Dr. Abdol Majid Kowsarinejad, Mr. Dr. Omid Jamshidi, Mr. Alireza Arab and all othe colleagues without whose helps and cooperations, it would be impossible to carry this study out.

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