VETERINARSKI ARHIV 76 (3), 217-225, 2006

Isolation of aerobic bacteria from the lungs of chickens showing respiratory disorders and confirmation of *Pasteurella multocida* by polymerase chain reaction (PCR)

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

ABSTRACT

In this study, lung samples of chickens slaughtered at an abattoir in Elazig province were tested for the presence of *Pasteurella multocida* and other aerobic bacteria. The identity of *P. multocida* was proved by mouse pathogenicity test and polymerase chain reaction (PCR). A total of 2000 chickens were examined at post-mortem, and 250 samples with pneumonia were collected. All lungs were collected from 10 different commercially reared chicken flocks showing respiratory disorders. Blood agar supplemented with 7% sheep blood was used for isolation of the agents. Of the examined chicken lung samples 16 (6.4%) *P. multocida* were isolated and identified. In addition, mouse pathogenicity test was carried out on *P. multocida* suspected isolates. Twelve (75%) of isolates were all positive. All *P. multocida*-suspicious isolates were positive in PCR. However, toxigenic *P. multocida* were not detected using PCR primers derived from toxA gene. This study showed that *P. multocida* is not widespread among the chicken population in Elazig.

Key words: *P. multocida*, polymerase chain reaction, chicken, lung

Introduction

Avian respiratory diseases represent serious economic losses in most poultry producing areas of the world. The aetiology of avian respiratory diseases caused mainly by bacteria, mycoplasma and viruses, is highly complex (TOTH, 2000). *Pasteurella multocida* has been consistently found in the upper respiratory tract, spleen, lungs, blood and liver of infected birds (RHOADES, 1964; HUNTER and WOBESER, 1980). Detection of *P. multocida* is important in the overall control and elimination of respiratory diseases from poultry

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ISSN 0372-5480
Printed in Croatia

ocks. Detection of *P. multocida* has mainly relied on mouse inoculation and *in vitro* culture in selective medium (CURTIS et al., 1981; BALDRIAS et al., 1988). Results from studies using selective media have been disappointing (GARLINGHOUSE et al., 1981). Some media will not support the growth of all possible isolates, whereas others are too insensitive (KASTEN et al., 1997). Mouse passage seems to represent a more efficient and widely accepted method of detecting *P. multocida*, although it may only select strains pathogenic for mice (BALDRIAS et al., 1988).

In recent years, genotypic methods of bacterial identification have proved beneficial in overcoming some limitations of traditional phenotypic procedures (TOWNSEND et al., 1998). Nucleic acid-based assays allow the detection of organisms directly from clinical samples or from small amounts of cultured bacterial cells, thus dramatically improving sensitivity and decreasing the time required for bacterial identification (TOWNSEND et al., 1998). PCR has been particularly useful in this regard, with use of primer sequences designed to facilitate identification at any level of specificity: strain, species, genus, or all members of a domain (RELMAN and PERSING, 1996). PCR is sensitive, specific and does not require laboratory animals (INNIS et al., 1990).

The gene that encodes the dermonecrotic toxin (DNT), toxA, has been isolated and sequenced (BUYS et al., 1990; KAMPS et al., 1990; LAX and CHANTER, 1990; PETERSEN, 1990). Hybridization studies using probes of the toxA gene showed its presence in all toxigenic *P. multocida* strains and absence in most nontoxigenic strains (KAMPS et al., 1990).

The objective of the present work was to isolate *P. multocida* and other bacterial agents from lung samples of chickens showing disorders of respiratory tract, slaughtered at a local abattoir, and to confirm identification for *P. multocida* by mouse pathogenecity test and a PCR method, and in addition, to investigate presence of toxA gene of *P. multocida* by PCR.

Materials and methods

Material. A total of 2000 chickens were examined for presence of pneumonia in lungs at a local abattoir in Elazig province located in the East of Turkey, and 250 samples were collected. The samples were obtained from ten different ocks. Samples were immediately transferred to the laboratory in sterile plastic bags, where they were processed.

Bacterial strains. P. multocida (obtained from Dr. L. Fodor, Department of Epizootiology, Hungary) and toxigenic *P. multocida* strains (kindly donated by Dr. E. M. Kamp, Institute for Animal Science Health, The Netherlands) used as positive controls, and distilled water used as negative control in PCR.

Culture. Swabs from the lung samples were inoculated onto blood agar supplemented with 7% sheep blood. The plates were incubated under aerobic conditions for 24-48 h at

37 °C. The agar plates were checked every day for suspected colonies. Identification or confirmation bacterial species was assessed by observation of the colonial morphology and Gram staining results and by biochemical methods. Methods were as follows: catalase, nitrate reduction, H_2S production in triple sugar iron (TSI), growth on MacConkey agar, Eosin Methylene Blue Agar, indol production, urease activity, metil red production, Voges Proskauer test reaction, oxidase reaction, coagulase, motility, citrate, carbonhydrate fermentation from glucose, trehalose, xylose, arabinose, fructose, galactose, maltose, mannose, sucrose, lactose, dulcitol, inocitol, salicin (CARTER, 1984).

Each isolate was frozen at -80 °C in a nutrient broth with 10% of glycerol for further analysis.

DNA extraction. Suspected *P. multocida* cultures were transferred into an Eppendorf tube containing 300 µL distilled water. The tubes were vortexed and incubated at 56 ºC for 30 min. The suspension was then added in 300 µL of TNES buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% SDS) and 200 µg/mL Proteinase K. Following 30 min boiling, an equal volume of phenol was added to the suspension which was shaken vigorously by hand for 5 min and then, centrifuged at 11.600 g for 10 min. The upper phase was transferred into a new eppendorf tube. Genomic DNA was precipitated with absolute ethanol and 0.3 M sodium acetate at-20 ºC for one hour, or overnight. The mixture was then centrifuged at 11.600 g for 10 min and the upper phase discarded. The pellet was washed twice with 300 µL of 90% and 70% ethanol, respectively; each step was followed by 5 min centrifugation. The pellet was dried and resuspended in 50 µL sterile distilled water and used as a target DNA in PCR.

Primers. Primers used were: PMOut1 (5'-AGGTGAAAGAGGTTATG-3') and PMOut2 (5'-TACCTAACTCAACCAAC-3') derived from Omp gene and PMTox1 (5'-GGTCAGATGATGCTAGATACTCC-3') and PMTox2 (5'-CCAAACAGGGTTATATTCTGGAC-3') (Promega) derived from *toxA* gene, respectively (NEUMANN et al., 1998; KAMP et al., 1996).

Polymerase chain reaction. PCR was performed in a Touchdown Thermocycler (Hybaid, Middlesex, England) in a total reaction volume of 50 µL containing 5 µL of 10x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton® X-100), 5 µL of 25 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate, 2U of Taq DNA Polymerase (Fermentas, Lithuania), 1 µM of each primer and 5 µL of template DNA. Amplification was obtained with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, and 56 °C for 1 min, and 72 °C for 2 min. Ten microlitres of PCR products were analysed on 1.5% agarose gel in 1XTris-Borate-EDTA (TBE) buffer. Gels were photographed under UV illumination after staining with 0.5 µg/mL ethidium bromide. Fragment sizes of 221 bp and 338 bp were verified as positive for *P. multocida* and toxigenic *P. multocida*, respectively. A 100 bp DNA ladder (Promega, Madison, WI, USA) was used as a molecular size standard.

Mouse pathogenicity test. Swiss-Webster mice used in this study were obtained from the Veterinary Control and Research Institute, Elazig-TURKEY. *P. multocida* colonies inoculated into nutrient broth. Mice (test mice) infected parenterally with $1x10^3$ CFU per mL of *P. multocida* strain. Control mice were also inoculated with phosphate-buffered saline. Both groups of mice were observed for 48 h. Control mice survived, while twelve test mice died at the end of this time. Heart blood and lung samples of died mice were stained with Gram and Giemsa staining and inoculated onto blood agar plates. The plates were incubated under aerobic conditions for 24-48 h at 37 °C and analysed for the presence of *P. multocida*. Suspected *P. multocida* colonies were subcultured. Isolates were identified by biochemical methods.

Results

Culture results. After 48 h of incubation on blood agar supplemented with 7% sheep blood under aerobic conditions for 24-48 h at 37 °C, grey to grey-white colonies, were observed. Biochemical reactions of isolates were typical of *P. multocida*. All strains

Table 1. Isolated bacteria and isolation rates

Isolated bacteria	N° of isolates (n)	The proportion (%)
Escherichia coli	47	18.8
Proteus spp.	40	16
Staphylococcus spp.	38	15.2
Streptococcus spp.	17	6.8
P. multocida	16	6.4
Corynebacterium spp.	10	4
Pseudomonas spp.	6	2.4
Yeast	4	1.6
Bordetella avium	3	1.2
E. coli + *Staphyloccocus* spp.	21	8.4
E. coli + *Proteus* spp.	2	0.8
Proteus spp. + *Staphyloccocus* spp.	2	0.8
Staphyloccocus spp. + *Streptococcus* spp.	2	0.8
E. coli + *Corynebacterium* spp.	1	0.4
Pseudomonas spp. + yeast	1	0.4
No growth	40	16
Total	250	100.0

presumed to belong to *P. multocida* were Gram negative rods, non-motile, produced oxidase, catalase, indol, presence of ornithine decarboxylase that fermented-mannitol, acid by fermentation of glucose and that did not grow in MacConkey agar.

Bacterial growth was observed in 210 out of 250 samples. The isolation of *Escherichia coli* was in a much higher percentage (18.8%) than that of *P. multocida* (6.4%). In addition, *Bordetella avium* was isolated from lung samples with a lesser percentage (1.2%) than other bacteria (Table 1).

PCR Results. PCR amplification of genomic DNA from *P. multocida* in this study using PMOut 1-2 and PMTox 1-2 primer pairs was obtained, corresponding to the anticipated sizes of 221 bp and 338 bp, respectively (Fig. 1). All *P. multocida* isolates that were positive by culture were also detected to be positive by PCR. In addition, no toxigenic *P. multocida* were detected in any of isolates by PCR. No amplified products were obtained from the negative control.

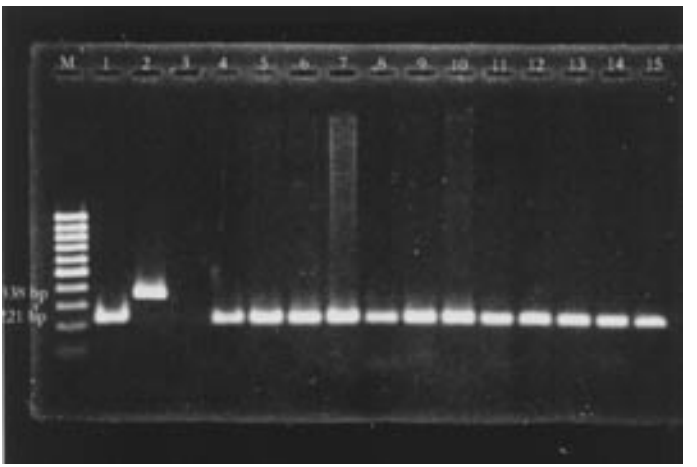

Fig. 1. PCR results of *P. multocida* isolates. (M: 100 bp DNA ladder, 1: positive control (*P. multocida* strain), 2: positive control (toxigenic *P. multocida* strain), 3: negative control, 4-15: *P. multocida* isolates.

Mouse pathogenicity results. Twelve (75%) *P. multocida* could be isolated from heart blood and lung samples of dead mice. However, *P. multocida* could not be isolated from four mice (25%) inoculated with strains that were both culture-and PCR-positive.

Discussion

Respiratory diseases are common among the chicken population. Various bacterial and viral pathogens are encountered in respiratory disease in domestic poultry (CHIN and

DROUAL, 1997). Isolation percentages obtained in this study were rather low considering the total number of animals examined. This may be due to the fact that most of our samples were not taken from ocks in the acute stage of infection, and also due to breeders in Turkey unconsciously using antibiotics in ocks. In addition, samples might be contaminated during the slaughtering processes.

Apart from *P. multocida*, *Staphyloccocus* spp., *Streptococcus* spp., *E. coli*, *Corynebacterium* spp., *Pseudomonas* spp., *Proteus* spp., yeast and *Bordetella avium* were isolated and identified from lung samples of chicken.

The mouse pathogenicity test is often used to determine the presence of *P. multocida* in samples contaminated with other microorganisms (QUAN et al., 1986). Adult male Swiss-Webster mice injected with approximately 10 or more *P. multocida* strain will succumb within 3 days (KASTEN, R. W. unpublished data). However, virulence for mouse has been reported to be variable (CURTIS et al., 1980). In a study comparing the mouse inoculation test with the PCR-based assay, they found that of the 23 samples positive by mouse inoculation, 14 (61%) were not detected by the PCR-H assay (KASTEN et al., 1997). In this study, *P. multocida* was not detected by mouse inoculation in four samples positive by PCR while culture was detected. It is possible that the four samples contained strains of *P. multocida* non-lethal for mice, taking into account that in the test less virulent strains are harder to detect when the mouse inoculation test is used (KASTEN et al., 1997).

Additional tests are needed to differentiate toxigenic and nontoxigenic *P. multocida* isolates. Both *in vitro* and in vivo methods for differentiating these two strains have been used (de JONG et al., 1980; NAGAI et al., 1994). However, culture isolation (AMIGOT et al., 1998), species identification (PIJOAN et al., 1984; CHOI and CHAE, 2001), and toxin testing (de JONG, 1980; PIJOAN et al., 1984; FOGED et al., 1988; AMIGOT et al., 1998) is time-consuming and costly (CHOI and CHAE, 2001). Recently, a PCR assay for differentiating toxigenic and nontoxigenic *P. multocida* was reported (NAGAI et al., 1994; LICHTENSTEIGER et al., 1996; KAMP et al., 1996; HOTZEL et al., 1997; NEUMANN et al., 1998). The PCR assay described by KAMP et al. (1996) was more sensitive than the other methods tested. TOWNSEND et al. (2000) indicated that detection of *P. multocida* from direct culture was less efficient than either mouse inoculation or PCR. Detection of *P. multocida* by PCR was particularly successful as some isolates were shown to be nonpathogenic for mice (TOWNSEND et al., 2000).

To date, several PCR tests have been described for *P. multocida* (KASTEN et al., 1997; TOWNSEND et al., 1998; MIFLIN and BLACKALL, 2001). Scientists demonstrated that primers derived from Omp and dermonecrotic toxin (toxA) gene serve for the differential identification of *P. multocida* and toxigenic *P. multocida* by the PCR (NEUMANN et al., 1998; KAMP et al., 1996). Primer pairs PMOut and PMTox proved to be specific for *P. multocida* and toxigenic *P. multocida*, respectively. All *P. multocida* strains that were positive by culture were also detected to be positive by the PCR. Toxigenic *P. multocida* was not isolated from any of these specimens.

This study showed that *P. multocida* is not widespread among the chicken population in Elazig. In addition, further studies are needed to understand the epidemiological importance of disorders of the respiratory tract in the chicken population of the region.

Acknowledgments

We thank Dr. L. Fodor, Department of Epizootiology, Hungary and Dr. E. M. Kamp, The Institute for Animal Science Health, The Netherlands for supplying *P. multocida* and toxigenic *P. multocida* strains, respectively.

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Received: 11 April 2005
Accepted: 2 May 2006

OZBEY, G., A. MUZ: Izdvajanje aerobnih bakterija iz pluća pilića s dišnim poremećajima i dokaz bakterije *Pasteurella multocida* lančanom reakcijom polimerazom. Vet. arhiv 76, 217-225, 2006.

SAŽETAK

Uzorci pluća pilića, zaklanih u jednoj klaonici u pokrajini Elazig u Turskoj, pretraženi su na prisutnost bakterije *Pasteurella multocida* i druge aerobne bakterije. *P. multocida* identificirana je pomoću testa patogenosti na miševima i lančanom reakcijom polimerazom (PCR). Ukupno je bilo pretraženo 2000 zaklanih pilića, a za bakteriološku pretragu uzeto je 250 uzoraka pluća pilića u kojih je ustanovljena pneumonija. Uzorci su bili prikupljeni s 10 peradarnika u kojima su pilići pokazivali dišne poremećaje. Krvni agar sa 7% ovčje krvi upotrijebljen je za izdvajanje bakterija. *P. multocida* bila je izdvojena iz 16 (6,4%) pretraženih uzoraka. Test patogenosti na miševima dodatno je proveden na izolatima sumnjivim na bakteriju *P. multocida*. Dvanaest (75%) izolata bilo je pozitivno tim testom. Svi izolati sumnjivi na vrstu *P. multocida* bili su pozitivni pretragom PCR. Međutim, toksigena *P. multocida* nije bila dokazana pomoću PCR-a upotrebom početnica podrijetlom od gena *toxA*. Istraživanje je pokazalo da *P. multocida* nije proširena u pilića na području Elaziga.

Ključne riječi: *P. multocida*, lančana reakcija polimerazom, pilići, pluća