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DETECTION OF TOXIGENIC PASTEURELLA MULTOCIDA BY THE POLYMERASE CHAIN REACTION (PCR) IN FIELD SAMPLES

BY

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SUMMARY

Pasteurella multocida is an important cause of pneumonia in newly born animals, usually as a secondary pathogen invading lungs injured by other bacteria or viruses. Toxigenic and notoxigenic Pasteurella multocida isolates can not be differentiated by morphology or standard biochemical reaction. A more rapid and accurate method to detect toxigenic Pasteurella multocida is needed for improving clinical diagnosis, prevention, treatment and epidemiological studies.

The feasibility of using polymerase chain reaction (PCR) amplification assay for accurate and rapid detection of toxigenic Pasteurella multocida in field samples was studied. The results showed that PCR assay could detect toxigenic Pasteurella multocida antigen in field samples. The results indicate that there was complete agreement of the toxigenic status of the Pasteurella multocida based on mouse lethality and PCR assays. The concordance of PCR results with the defined toxigenic status indicates 100% specificity and sensitivity . The PCR protocol could be completed within one working day which is faster and more sensitive than the time consuming conventional diagnostic techniques currently used in clinical laboratories.

INTRODUCTION

Pasteurella multocida is a part of the commensal flora on the mucous membranes of the upper respiratory tract of numerous animal species. Pasteurella multocida induces pneumonia usually as a secondary pathogen invading lungs injured by other bacteria or viruses (Rimler and Rhoades, 1989). Toxigenic Pasteurella multocida isolates synthesize a 145-kDa toxin [dermonecrotic toxin (DNA] encoded by the chromosomal tox A gene (Petersen and Foged, 1989). Toxin-producing Pasteurella multocida organisms are involved in the etiology of naturally occurring progressive atrophic rhinitis of goats, cattle, rabbits, cats, dogs, pigs and turkeys (Kielstein, 1986; Nielson et al., 1986; Rimler and Brogden, 1986; Baalsrud, 1987 and Rhoades and Rimler, 1990).

Nontoxigenic Pasteurella multocida isolates do not cause this disease (Rutter and Rojas, 1982).

Toxigenic and nontoxigenic Pasteurella multocida isolates do not differ on diagnostic biochemical reaction or morphology.Pasteurella multocida toxin can be detected in cell extracts and culture filtrates by its lethality and lienotoxicity for mice (Nakai et al., 1994) and production of dermonecrosis in guinea pig skin (De Jong, 1992). The discriminative properties of these tests all depend on the detection of the DNT protein or its activity. Recognition of toxin-producing organisms by these methods is time-consuming, and the number of samples that can be procesed is limited (De Jong, 1992). In Vitro techniques such as cytopathogenic effects in tissue culture (Rutter and Luther, 1984), the agar overlay method (Chanter et al., 1989), and an enzyme-linked immunosorbent assay with toxin-specific monoclonal antibodies (Foged et al., 1988) can recognize toxin-producing Pasteurella multocida. While these in vitro techniques allow testing of a large number of suspect toxin-producing Pasteurella multocida. isolates, they are cumbersome and require expensive equipment.

A more rapid, accurate detection assay is needed for sound decisions regarding diagnosis and treatment, to prevent unintentional introduction of infected animals into clean herds, to support basic studies in ecology and epidemiology of the organism, and to develop more efficacious vaccines. Assays based on PCR are contributing to diagnostic microbiology (Eisenstein, 1990). The conservation of the sequence of the toxA gene (Buys et al., 1990 and Petersen, 1990) indicates that assays for the gene, including PCR, are valid for identification of toxigenic isolates. Detection of toxigenic Pasteurella multocida. using PCR should be a more rapid and sensitive assay than the three-step process of bacterial isolation, biochemical identification, and toxigenic testing of isolates. The objective of this study was to evaluate the PCR assay for detection of toxigenic Pasteurella multocida.

MATERIAL AND METHODS

Pasteurella multocida:

Two toxigenic (one type A and one type D) and two nontoxigenic (one type A and one type D) Pasteurella multocida strain were used as assay control;s. All strains were kindly supplied by Prof. Dr. More, Arizona state University, USA.

Handling of swabs:

One hundred and seventy five nasopharyngeal swabs from pneumonic kids, cattle and buffalo-calves were collected. Plastic-shifted swabs were used without moistening the tip with the builty-in transport medium. The tips of all swabs were excised into 1.8-ml centrifuge tubes. Stock samples suspensions were prepared by adding 0.5-ml aliquot of transport medium in each tube, and the tubes were manually inverted a few times.

Isolation and identification of Pasteurella multocida.:

Isolation and biochemical identification of Pasteurella multocida from the stock sample suspensions was done according to the methods described by Finegold and Martin (1982). Capsular typing of Pasteurella multocida isolates was performed by using indirect haemagglutination test according to Carter and Rappay (1962). The toxigenic status of the isolates was defined according to the methods described by Nakai et al. (1994).

Oligonucleotides

The oligonucleotide primers were designed to amplify and 846-mer segment of ToxA between nucleotides 2096 and 2942; the forward primer was 5' CTTAGATGAGGGACAAGG3', and the reverse primer was 5' GAATGCCACACCTCTATAG3', (Petersen, 1990). The specific primers were synthesized using DNA synthesizer [Instiutute for Molecular Biology and Genetic Engineering, ARC, Egypt].

Primer-directed amplification:

For PCR assay, control Pasteurella multocida strains and stock sample suspensions were grown to mid-to late log phase in brain heart infusion broth, pelleted by centrifugation and resuspended in 50µl of sterile distilled water. Template DNA was prepared by heat lysis (50 µl of bacteria incubated in boiling water for 10 minutes) (Maniatis et al., 1984).

PCR assays were run with 0.5 µl aliquots of template DNA in a 50-µl reaction volume. Taq polymerase (10µl) with reaction buffers (Gibco BRL Life Technologies, Grand Island, N.Y.), 0.4 mM nucleotides (Promiga Corp., Madison, Wis.), and synthesized primers (0.2µM) were used. The PCR mixture were overlaid with 40 µl of paraffin oil (Sigma, St. Louis, Mp., USA). The samples were subjected to 40 cycles of amplification in a Thermocycler (TempCycler II Modles 11 OP and 11OS Coy corporation, Grass Lake, Michigan, USA). The cycling conditions were as follows: Denaturation, 1 minute at 94°C; primer annealing 1 minute at 55°C; and extension, 1 minute at 72°C (Atlas and Bej, 1994 and Altwegg and Verhoef, 1995). To identify false-positive results, negative control reactions were used.

Identification of the PCR products:

Following amplification, a 5 µl of the PCR product was mixed with 5µl of x gel loading buffer and taken for electrophoresis on a 2% (w/v) agarose gel containing 0.5µl/ml ethidium bromide. The samples were electrophoresed at 100 volt for 1.5 to 2 hours in Bio-Rad electrophoresis unit (Maniatis et al., 1984). The presence of specific bands was detected by visualization with UV light and compared with molecular size marker.

RESULTS

Validity of PCR for Pasteurella multocida:

To be valid, the PCR assay must be specific for the toxA gene of Pasteurella multocida applicable to all toxigenic Pasteurella multocida isolates and sensitive enough to detect just a few organisms.

The figure shows the results of DNA amplification of four Pasteurella multocida reference strains (toxigenic A and D types as well as nontoxigenic A and D type) with the PCR assay using the toxA primer set. The expected amplicon was readily detected in the reaction product of toxigenic Pasteurella multocida strains resolved in agarose gel and stained with ethidium bromide (Lanes, 1 and 8). Similar product was not synthesized when nontoxigenic Pasteurella multocida strains provided the template DNA (Lanes, 26 and 27).

The results indicated that 21 Pasteurella multocida isolates were isolated and identified out of 175 nasopharyngeal swabs collected from pneumonic kids, cattle and buffalo-calves. To determine the accuracy of PCR detection of toxigenic Pasteurella multocida in field samples, all positive swabs by using conventional isolation methods weere screened by the PCR and mouse lethality assays to define true positive (toxigenic) and true negative (nontoxigenic) Pasteurella multocida. The table and the figure show the results of isolation of Pasteurella multocida, capsular typing , toxigenic status of these isolates and PCR assay.

Sensitivity of PCR amplicon detection was studied. The figures shows a signal from 102 CFU from type D and type A Pasteurella mul;tocida strains (lanes, 2 and 9) was visible on the ethidium bromide stained gel under ultraviolet light.

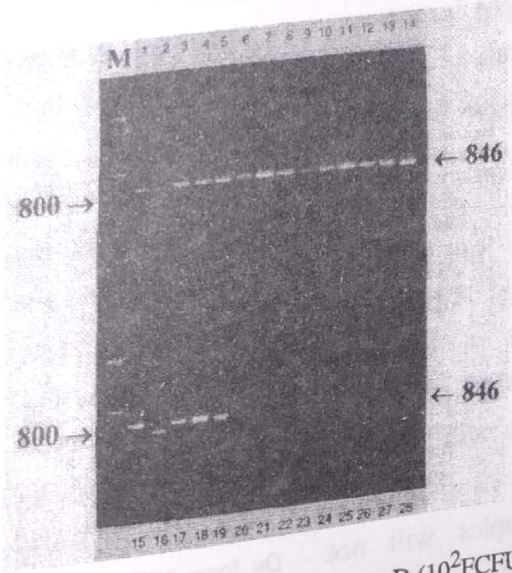
DISCUSSION

Accurate and rapid detection of toxigenic Pasteurella multocida is important for the intellight interpretation of epidemiological data. Moreover, an efficient pasteurellosis program of prevention or control depends on reliable epidemiological information. Because of its potential to detect very small numbers of organisms, PCR assay has been applied to the diagnosis of a number of infections, particularly viruses, that had previously caused diagnostic difficulty (Saiki et al., 1988). Recent work has also demonstrate its practical application in bacterial infection in Egypt (Amin, 1995 and Amin et al., 1995).

The results indicated that 21 Pasteurella multocida strains were isolated and identified out of 175 nasopharyngeal swabs collected from pneumonic kids , cattle buffalo-calves. To evaluate the PCR for detection of toxigenic Pasteurella multocida in field samples, . All positive swabs by using conventional methods were screened by the PCR and mouse lethality assays to define true positive (toxigenic) and true negative (nototoxigenic) Pasteurella multocida. The table and the figure show that 11 of 14 type D and 3 of 7 type A isolates were defined toxigenic by the PCR assay, and all other isolates were nontoxigenic. The results indicates that there was complete agreement of the toxigenic status of the Pasteurella multocida based on mouse lethality and PCR assays. The concordance of PCR results with the defined toxigenic status indicates 100% specificity.

PCR and Mouse lethality toxigenic assay results from diagnostic Pasteurella multocida

Sample No.	Capsule type	Mouse lethality assay	PCR
1	D	Positive	Positive
2	D	Positive	Positive
3	D	Positive	Positive
4	D	Positive	Positive
5	D	Positive	Positive
6	D	Positive	Positive
7	D	Positive	Positive
8	D	Positive	Positive
9	A	Positive	Positive
10	A	Positive	Positive
11	A	Positive	Positive
12	D	Negative	Negative
13	D	Positive	Positive
14	D	Positive	Positive
15	D	Positive	Positive
16	A	Negative	Negative
17	D	Negative	Negative
18	A	Negative	Negative
19	D	Negative	Negative
20	A	Negative	Negative
21	A	Negative	Negative



Lane 1, toxigenic type D (10^5 CFU); :lane^2, toxigenic type D (10^2FCFU); Lane 8, toxigenic type A (10^5 CFU); Lane 9, toxigenic type A (10^2FCFU); Lane 26, nontoxigenic type D; Lane 27 ,non toxigenic type A; Lanes 3,4,5,6,7,10,11,12,16,17,18,19,21 and 23 Type D isolates; Lanes 12,14,15,20,22,24 and type A isolates; Lane 28, negative control (no DNA) and M, marker.

The results from the four Pasteurella multocida control strains (toxigenic types A and D and nontoxigenic types A and D) and field samples indicates that the reactivity is not restricted by variability of toxA gene among isolates or capsule. We came to the same conclusion as by Nakai et al. (1994) that toxA is in the same location in the chromosome of both capsules A and D of Pasteurella multocida

Primers are critical to sensitive PCR results (Wang et al., 1994); the apparent increased sensitivity of this PCR protocol (about 10^2 CFU) over that previously reported by Rolfs et al., 1992 (about 10^3 CFU) , assuming 4.6g of DNA per bacterium) is likely due to primer set differences.

In addition to validity, minimal cost and ease of performance are important for both diagnostic and high-volume research assays such as epidemiological studies. This PCR protocol is less labor-intensive and avoids hazardous chemicals used in other protocols in which DNA is extracted or the sample digested with proteinase K prior to PCR. Boiling is all that was needed prior to PCR amplification.

Considering the available data, we can conclude that the PCR technique is useful and sensitive for research or diagnostic purposes of toxigenic Pasteurella multocida in clinical samples. Development of a rapid, specific and sensitive assay for detection of toxigenic Pasteurella multocida in nasal swabs samples will not facilitate rapid clinical diagnosis and prompt therapy but will also facilitate epidemiological studies and screening to prevent transmission to clean herds by animal movement.

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