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RESEARCH ARTICLE

GENOTYPIC AND BIOCHEMICAL CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* ISOLATED FROM SHEEP AT SLAUGHTER IN AMMAN-JORDAN

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ABSTRACT

Pasteurella multocida (p.m.) is a gram negative coccobacilli bacterial pathogen, classified into three subspecies, five capsular serogroups, which is the causative agent of a range of diseases in domestic animals of economic significant worldwide. The aims of this study are isolation of *P. multocida* from sheep and identification by biochemical tests and polymerase chain reaction technique (PCR) technique. Seventy samples were isolated from sheep lung lesions and were cultured for *P. multocida* using conventional method and isolated colonies were subjected to biochemical tests and for identification by PCR. The results in the present study showed that the biochemical tests provided an approximate 99.9% (an excellent identification for *P. multocida*). The PCR analysis applied on direct bacterial colonies using specific primer gave an amplified product (219 bp) specific for *P. multocida*, this result confirmed the results of biochemical tests and provided obvious results for the identification of this bacteria using the specific primer PMout for omp gene of *P. multocida*. The PCR assay was found to provide rapid and accurate diagnosis of *P. multocida* isolates obtained from sheep respiratory infections during the investigation of suspected hemorrhagic septicemia outbreaks. PCR technique is recommended to be used for identification procedure to be applicable in all veterinarian laboratories for bacterial identification; likewise the production of veterinary vaccines productions companies. In addition RAPD technique is reliable means of discriminating between *P. multocida* isolates obtained from sheep.

Key words: Biochemical tests, *Pasteurella multocida*, PCR analysis, Genotyping.

INTRODUCTION:

Pasteurella is a genus of gram negative coccobacilli fucltatively anaerobic bacteria belong to Pasteurellaceae family. Pasteurellaceae are gamma proteobacteria the largest subgroup with extraordinary variety of physiological types [1]. *P. multocida* is responsible for wide range of diseases in domestic animals of economic significance, cattle, buffaloes, sheep, goats, rabbits and poultry throughout the world where outbreaks usually lead to high mortality and great economic loss to the ruminant industry [2]. This bacteria is also responsible for human infections ([3], [4], [5]). *Pasteurella* produces a number of proteins and polysaccharides that are thought to be important to its virulence. These factors have been identified by immunological and biochemical characterization of *P. multocida* gene product ([6], [7], [8], [9]). These are endotoxin, leukotoxins, capsular polysaccharides, sialoglycoproteinase and nuraminidase. It was documented that the molecular basis of toxin activity is the deamidation of glutamine residue leading to activation of alpha subunit of heterotrimic G- proteins that is essential for GTP hydrolysis, which stimulate diverse cellular signal transduction pathways ([10], [11].

Potential virulence factors have been reviewed [12]. More recently, Verma and his colleagues indicated that there are eleven virulence associated genes by PCR and reported the virulence associated gene ptfA is a positive association with disease outcome in cattle [13].

Moreover, *P. multocida* toxins were found to possess many properties such as a potential carcinogenic, a highly potent mitogen and blockade apoptosis ([14], [15]). However, *P. multocida* is a causative agent of many major diseases in animals causing haemorrhagic septicaemia in cattle and buffaloes, pneumonic septicaemic pasteulellosis in sheep and goats, atropic rhinitis in pigs, snuffles in rabbits and fowl cholera in poultry [16].

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Human infections are, in most cases, of animal origin and most often related to bites or scratches by carnivores, through other types of infections have also been reported [17].

MATERIALS AND METHODS:

1**. Bacterial isolates; growth conditions and species confirmation:**

Seventy samples were collected from lung lesions of imported sheep from Amman province slaughterhouse in Jordan. Isolates were cultured on Blood Agar and MacConky agar and incubated for 24 hours at 37^{o} C. According to morphological analysis, the susceptible colonies isolates were sub cultured and tested for oxidase activity; positive isolates were confirmed by Gram stain.

To identify the bacterial strain, AP® 20E test strip (BioMerieux, Inc.) for Gram negative bacteria was used. Twenty dehydrated separate test compartments were on the strip. A bacterial suspension is used to rehydrate each well. Some of the wells had color changes due to pH differences; others produced end products that needed to be identified with reagents. A profile number was determined from the sequence of (+) and (-) test results, and then looked up in a codebook having a correlation between numbers and bacterial species.

2. Genomic DNA Analysis:

The nine pure cultures used for DNA extractions according to [2]. A few colonies from bacterial cultures were transferred into an eppindorf tube containing 300 µl distilled water. The tubes were mixed and incubated at 56°C for 30 minutes. The suspension was then mixed with 300 µl of K-buffer (20mM Tris pH 8.0 + 150 mM NaCl + 10 mM EDTA + 0.2%SDS) and 200 µg/ml proteinase K. Following 30 minute boiling at 65°C, an equal volume of phenol was added to the suspension that was shaken vigorously by hand for 5 min and then, centrifuged at 14000 rpm 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 and kept at -20 °C over night. The mixture was then centrifuged at 14000 rpm for 10 min and the upper phase was discarded. The pellet was washed with 300 µl of 70% ethanol, and was dried at room temperature. The pellets were resuspended in 25 µl sterile distilled water and used as a template DNA in PCR [18].

3. Agarose gel electrophoresis:

From each sample, 5 µl was mixed with 1 µl of loading dye and applied on the agarose gel 0.7% (w/v) with ethidium bromide for 30 minutes and 150 volt for genomic DNA.

4. **PCR Analysis:**

The identification of *P. multocida* was confirmed by PCR using PMOut primers; forward (5'-AGG TGA AAG AGG TTA TG-3'), reverse (5'-TAC CTA ACT CAA CCA AC-3') (Ozeby et al., 2004). The PCR reaction was performed in a total volume of 25 µl consisting of 1 µl template DNA, 5x PCR buffer, 25 mM $MgCl_2$, 10 µM deoxynucleoside triphosphates, 1,25 U of Taq DNA polymerase. PCR cycling program was as follow: 41 cycles of 94 °C for 45 seconds, 45 °C for 45 seconds, and 72 °C for 1 min. A final step of extension was applied at 72 °C for 5 min. Ten microliters of PCR products were analyzed by electrophoresis in a 1.0% agarose gel with Tris Borate EDTA buffer pH 8.0.

RESULTS AND DISCUSSIN:

1. Microbial Analysis:

The phenotype analysis was used to differentiate bacterial isolates, at that time. The two bacterial cultures were omitted because the morphology of the cultivated colonies was not *Pasteurella*. The remaining bacterial cultures were subcultured on Blood agar and MacConky agar to obtain pure colonies. After 24 hours, susceptible colonies were tested by oxidase test; twenty three samples of the cultured bacteria gave positive results for oxidase test. Then the bacterial colonies that showed positive oxidase test were stained by Gram's stain; fourteen cultures were omitted and none pure cultures were confirmed because they revealed gram negative and coccobacillus. These nine positive isolates were examined using API® 20E strip test.

2. Biochemical test:

According to Table (1), the profile number was determined from the sequence of positive (+) and negative (-) test results. Samples of 1, 2, 6, and 7 had the same code (0140520), samples of 3, 4, 5, and 8 had the same code (0040520) by looked up in a codebook that having a correlation between numbers. These two profile numbers provided an excellent identification for *P. multocida* type 2 with identification percentage of 99.9%. The API® 20E results was then confirmed by PCR.

Table 1: Results of nine bacterial cultures according to API® 20E biochemical test

Test	Substrate	Reaction tested	Samples								
			1	2	3	4	5	6	7	8	9
ONPG	ONPG	Beta galactosidase	-	-	-	-	-	-	-	-	+
ADH	arginine	Arginine dihydrolase	-	-	-	-	-	-	-	-	-
LDC	Lysine	Lysine decarboxylase	-	-	-	-	-	-	-	-	-
ODC	Ornithine	Ornithine decarboxylase	+	+	-	-	-	+	+	-	-
CIT	Citrate	Citrate utilization	-	-	-	-	-	-	-	-	-
H_2S	Na thiosulfate	H_2S production	-	-	-	-	-	-	-	-	-
URE	Urea	Urea hydrolysis	-	-	-	-	-	-	-	-	-
TDA	Tryptophan	Deaminase	-	-	-	-	-	-	-	-	-
IND	Tryptophan	Indole production	+	+	+	+	+	+	+	+	+
VP	Na pyruvate	Acetoin production	-	-	-	-	-	-	-	-	-
GEL	Charcoal gelatin	Gelatinase	-	-	-	-	-	-	-	-	+
GLU	Glucose	Fermentation/oxidation	-	-	-	-	-	-	-	-	-
MAN	Mannitol	Fermentation/oxidation	+	+	+	+	+	+	+	+	-
INO	Inositol	Fermentation/oxidation	-	-	-	-	-	-	-	-	-
SOR	Sorbitol	Fermentation/oxidation	+	+	+	+	+	+	+	+	-
RHA	Rhamnose	Fermentation/oxidation	-	-	-	-	-	-	-	-	-
SAC	Sucrose	Fermentation/oxidation	+	+	+	+	+	+	+	+	-
MEL	Melibiose	Fermentation/oxidation	-	-	-	-	-	-	-	-	-
AMY	Amygdalin	Fermentation/oxidation	-	-	-	-	-	-	-	-	-
ARA	Arabinose	Fermentation/oxidation	-	-	-	-	-	-	-	-	-

3. PCR analysis:

PCR identification by specific primers was applied to ensure and confirm the biochemical test results. The specific primer recognized the specific sequence on the genomic DNA. The results of PCR of the different DNA samples showed amplified bands of identical size (219 bp) figure 1. These results confirmed the results of the biochemical tests. All positive cultures for biochemical tests were also PCR positive. In fact, the biochemical tests provided an approximate 99.9% (excellent identification) for *P multocida* type 2, and PCR analysis confirmed these results and provided an obvious results for the identification of this bacteria using the specific primer PMOut.

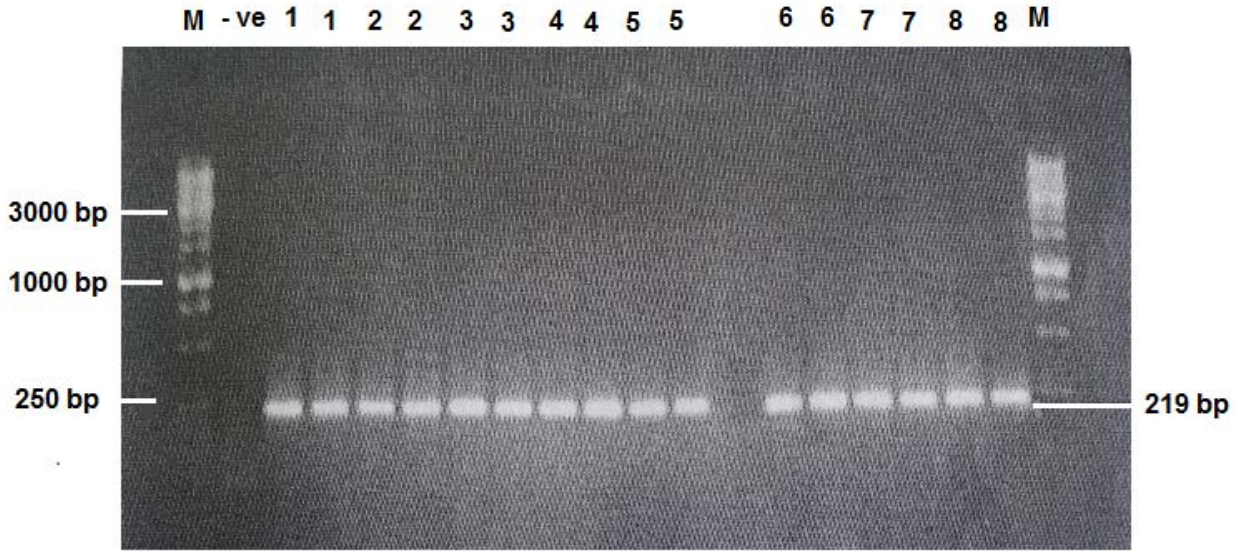

Figure 1: Ethedium bromide-stained agarose gel (1% w/v) showing positive results of PCR amplification of the *Omp* gene in eight samples of P. multocida. Two replicates of each sample of (1, 2, 3, 4, 5, 6, 7, and 8) were generated ofbacterial isolates. Lane M (1 kb marker) and (- ve) is negative control.

The traditional methods of identifying *Pateurella* species are often based on examination of phenotypic, morphologic characteristics [19]. This approach can be time consuming and reliance on the variable expression of phenotypic characteristics can lead to inconsistent results. Recently, molecular methods which are based on genomic characteristics such as RAPD-PCR have been used for classification and discrimination of *Pasteurella multocida* isolates.

The PCR assays were shown to be species specific providing a valuable supplement to phenotypic identification. Many researchers subjected isolates *P. mulotcida* from different animals to phenotypic characterization and PCR analysis. They documented that PCR amplification directly on bacterial colonies was an extremely rapid, accurate, and sensitive method of *P. multocida* ([2], [20], [21], [22]). The result of our study are quite similar to those obtained in these reports and show that there was 100% agreement and consistency between phenotypic analysis and genotypic analysis by PCR technique, which demonstrates that this method can be applied to directly identified *Pasteurella* spp. And our results are supported by above author's results.

In conclusion, RAPD analysis is not only faster and simple to use but additionally useful given its consistency with conventional methods. When specific molecular biology equipment and trained personnel are lacking (e.g. slaughter), the phenotypic systems remain the first choice for *P. multocida* identification.

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