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Original Research

Molecular versus conventional culture for detection of respiratory bacterial pathogens in poultry

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Abstract: Acute respiratory tract infections are leading causes of morbidity in poultry farms allover the world. Six pathogens; *Escherichia coli, Mycoplasma gallisepticum, Staphylococcus aureus, Pasteurella multocida, Mannheimia haemolytica* and *Pseudomonas aeruginosa* were involved in respiratory infections in poultry. Herein, conventional identification procedures and polymerase chain reaction (PCR) were applied for detection of the most common respiratory bacterial pathogens in clinical specimens of poultry obtained from 53 Egyptian farms with various respiratory problems and the results were compared statistically. The analyzed data demonstrated a significantly higher rate of detection of the most recovered microorganisms ($P < 0.05$) by PCR comparing to classical culture procedures. Further, multiplex PCR could detect *E. coli*, *M. gallisepticum, S. aureus* and *Ps. aeruginosa* in a single reaction, however, *M. haemolytica* was reported in a uinplex system. According to PCR results, the most commonly recorded bacterial pathogens in examined poultry farms were *E. coli* and *Ps. aeruginosa* (54.71% each), followed by *M. haemolylica* (35.85%) and *M. gallisepticum* (20.75%). In conclusion, PCR assay offered an effective alternative to traditional typing methods for the identification and simultaneous detection of the most clinically relevant respiratory pathogens in poultry.

Key words: Poultry diseases, respiratory pathogens, bacterial isolation, Multiplex PCR.

Introduction

Acute respiratory tract infections are of paramount importance in the poultry industry worldwide (1). A broad variety of pathogens have been associated with respiratory infections in poultry comprising viruses (Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), etc), bacteria (*Mycoplasma* species, *Haemophilus paragallinarum, E. coli*, ornithobacterium, several microorganisms of the genus pasteurella including *P. multocida*, *P. gallinarum, P. anatipestifer* and *M. haemolylica* as well as fungi and parasites (2, 3). These pathogens can cause a disease independently, in association with each other, or in association with other bacterial or viral agents (4). Environmental factors may augment these pathogens to produce the clinically observed signs and lesions (5). Indeed, the use of standard culture methods for detecting of bacterial pathogens is cumbersome and time-consuming. During the last decades, the introduction of less time-consuming and more sensitive molecular techniques, such as PCR assays (6, 7), has contributed significantly in diagnosis of infections. Moreover, the application of multiplex PCR assays for simultaneous identification of several bacterial pathogens seems to be reliable, rapid, and cost effective (8, 9). Using this highly sensitive and specific methodology, early information could be obtained about the presence of the targeted pathogens in the animal population. Furthermore, the emergence of new variants could be detected and the efficiency of immunizations and/or medications could be monitored; thereby, controlling measurements could be started on time (10). Herein, this approach aimed to evaluate PCR assay, particularly multiplex PCR, in comparison to conventional culture techniques for detection of the most common respiratory bacterial pathogens in clinical specimens of poultry with various respiratory problems, further, to report the prevalence of these pathogens in our Egyptian poultry farms.

Materials and Methods

Clinical specimens

Two hundred and fifty specimens of tracheal tissues and lungs collected from 53 Egyptian poultry farms (50 chicken farms of different production sectors, 2 duck farms and an ostrich farm) with various forms of respiratory problems were analyzed. Tissue specimens of each farm were divided into 2 parts; one part was placed into a sterile separate container and transported to the laboratory in an ice box within 24 h for bacteriological examination, the other one was pooled into approximately 1 ml of buffered peptone water (Oxoid, UK) and stored at -80°C to be examined directly by PCR.

Isolation and identification of avian respiratory bacteria

Pleuropneumonea like organism (PPLO) broth and agar base media (Difco, USA) supplemented with mycoplasma selective supplement G (SR0059, Oxoid, Hampshire, England, UK) were used for mycoplasma isolation as described elsewhere (11). Conventional identification of mycoplasma depended mainly on digi-

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Table 1. Target genes, oligonucleotide primers and their amplicons used in PCR.

Target gene	Primer sequence (5'-3')	Annealing temperature (°C)	Amplicon (bp)	Reference
*E. coli pho*A	F: CGATTCTGGAAATGGCAAAAG R: CGTGATCAGCGGTGACTATGAC	55	720	(15)
M.gallisepticum 16S rRNA	F:GAGCTAATCTGTAAAGTTGGTC R:GCTTCCTTGCGGTTAGCAAC		185	(16)
*S. aureus clf*A	F: GCAAAATCCAGCACAACAGGAAACGA R: CTTGATCTCCAGCCATAATTGGTGG		638	(17)
*Ps. aeruginosa opr*L	F: ATGGAAATGCTGAAATTCGGC R: CTTCTTCAGCTCGACGCGACG		504	(18)
P. multocida 23S rRNA	F:GGCTGGGAAGCCAAATCAAAG R: CGAGGGACTACAATTACTGTA A	69	1432	(19)
M. haemolytica ssa	F: TTCACATCTTCATCCTC R: TTTTCATCCTCTTCGTC	45	500	(20)

tonin sensitivity (12), glucose fermentation, and arginine deamination tests (13).

For isolation and identification of bacteria other than mycoplasmas, 10% sheep blood agar, trypticase soya agar (Difco, USA), Baired Parker agar (Oxoid, UK), MacConkey`s agar (Oxoid, UK), and eosin methylene blue agar media (Oxoid, UK) were used (14)**.** For biotyping, fresh colonies of presumptive bacterial growths were further identified with analytical profile index (API-20) identification kits (BioMérieux, Mary l'Etoile, France). O-serogrouping of *E. coli* isolates was applied in the Serology Unit, Animal Health Research Institute, Giza, Egypt, using commercial antisera (Difco, Detroit, MI, USA) according to the manufacturer's instructions.

DNA extraction

Genomic DNAs were extracted directly from respiratory organs of diseased birds using commercially available kit, QIAamp DNA Mini Kit, Catalogue no.51304 (Qiagen; Germany) according to manufacture's instructions.

Primer specificity

Oligonucleotide primer sets that specifically amplify the target sequences of *E. coli* alkaline phosphatase (*pho*A), *M. gallisepticum* ribosomal RNA (*16S* rRNA), *S. aureus* encoding a surface-associated fibrinogen-binding protein (*clf*A), *Ps. aeruginosa* outer membrane lipoprotein (*opr*L), *P. multocida* ribosomal RNA (*23S* rRNA) and *M. haemolytica* serotype specific antigen (*ssa*) genes are listed in Table 1. Virtual primers specificity was checked in a BLAST search available through the National Centre for Biotechnology Information (NCBI), website www.ncbi.nlm.nih.gov, before they were synthesized at Bio Basic, Canada INC. Further, six reference strains of *E. coli*, *M. gallisepticum*, *S. aureus*, *Ps. aeruginosa*, *P. multocida* and *M. haemolytica* previously isolated from poultry source during routine diagnostic examinations then fully identified, were used for testing the specificity of primers as well as positive controls. They were kindly provided by National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Giza, Egypt.

PCR procedures

Multiplex PCR assay was simultaneously carried out in triplicates for detection of four pathogens namely, *E. coli, M. gallisepticum, S. aureus* and *Ps. aeruginosa*. The optimal PCR amplification reaction mixture contained 37.5 *µl* Emerald Amp GT PCR master mix (2x premix), 13.5 *µl* PCR grade water, 5 *µl* of each primer (20 pmol), and 18 *µl* template DNA in a final volume of 79 *µl*. PCR amplification was performed in T3 thermal cycler (Biometra, Germany) with the following cycling conditions: initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s; primer annealing at 55 °C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The annealing temperature was empirically optimized to give the best specificity. However, *P. multocida* ribosomal RNA (*23S* rRNA) and *M. haemolytica ssa* genes were detected in uniplex PCR systems. Positive controls (reference strains) and negative controls (containing no DNA) were included in each assay run.

The amplified PCR products were electrophoresed on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x tris-boric EDTA buffer. A 100 bp DNA ladder (Qiagen, Germany, GmbH) was used as a molecular weight marker. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra, Germany) and the data were analyzed through computer software [BioDoc Analyze Digital Systems (Biometra, Germany)].

Statistical Analysis

The paired proportion McNemar's Chi-squared analysis was used through cross table procedure of the Statistical Package for Social Sciences (SPSS) version 21.0 (IBM Corp., Armonk, NY, USA) in order to compare the results of PCR assays to those of culture. *P* values of < 0.05 were considered statistically significant.

Results

Bacteriological examination of 53 Egyptian poultry farms suffered from respiratory manifestations by standard culture-based techniques showed that *E. coli* was highly recorded with a percentage of 47.17%. The most prevalent serotypes were O127:K63 and O44:K74 (16% each) followed by O119:K69, O164:K- and O118:K- (12% each), other infrequently encountered serotypes were included (O114:K90, O111:K58 and O158:K-). Moreover, *M. haemolytica*, *Ps. aeruginosa*, *M. gallisepticum* and *S. aureus* were also recorded with percentages of 20.75%, 15.09%, 5.66% and 3.77%, respectively.

In current study, uniplex PCR amplifications of *E. coli pho*A, *M.gallisepticum 16S* r*RNA, S. aureus clf*A, *P. multocida* 23S rRNA, *M. haemolytica ssa* and *Ps. aeruginosa opr*L genes revealed amplicons of expected

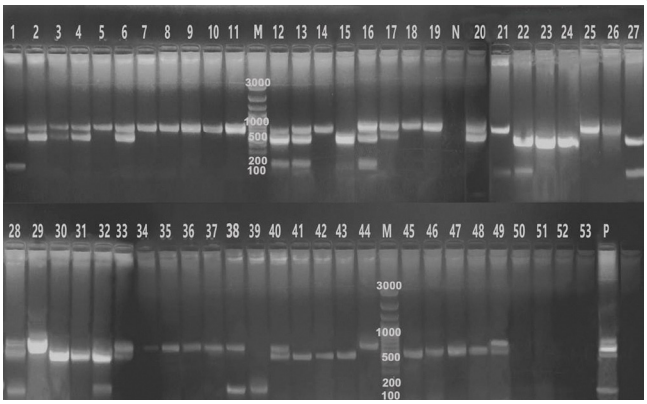

Figure 1. Agarose gel electrophoresis showing predicted PCR products for the amplified genes of some respiratory bacteria in clinical specimens of poultry using a multiplex PCR system. Lanes 1, 13, 16: *E. coli*, *Ps. aeruginosa* and *M. gallisepticum* (720, 504 and185 bp, respectively); lanes 2-4, 6, 17, 40, 49: *E. coli* and *Ps. aeruginosa* (720 and 504 bp, respectively); lanes 5, 7-11, 14, 18, 19, 25, 26, 29, 34-37, 44: *E. coli* (720 bp); lanes 12, 28: *S. aureus, Ps. aeruginosa* and *M. gallisepticum* (638, 504 and 185 bp, respectively); lanes 15, 23, 24, 30, 31, 41-43, 45-48: *Ps. aeruginosa* (504 bp); lanes 20, 33: *S. aureus* and *Ps. aeruginosa* (638 and 504 bp, respectively); lanes 21, 38: *E. coli* and *M. gallisepticum* (720 and 185bp, respectively); lanes 22, 27, 32: *Ps. aeruginosa* and *M. gallisepticum* (504 and 185 bp, respectively); lane 39: *M. gallisepticum* (185 bp); lanes 50-53: negative farms; lane N: negative control; lane P: positive control; lane M: molecular size marker (100 bp).

sizes indicating specificity of primers to those analyzed reference strains (data not shown). Besides, results of multiplex PCR on clinical specimens of the 53 Egyptian poultry farms showed that both *E. coli* and *Ps. aeruginosa* were equally identified with a percentage of 54.71% followed by *M. gallisepticum (*20.75%) and *S. aureus (*7.55%) (Figure 1). The reliability of the test was comparable to existing culture-based techniques. Obviously, multiplex PCR assay has the potential to be a rapid, accurate, specific and highly sensitive molecular diagnostic tool for simultaneous detection of avian respiratory bacteria within 5 to 6 hours. It could demonstrated a significantly higher rate of detection of the most recovered microorganisms ($P < 0.05$) from poultry farms with various respiratory problems than that was recorded by classical culture procedures (Table 2).

Further, uniplex PCR could detect *M. haemolytica ssa* gene in clinical specimens of the concerned farms with a percentage of 35.85% (19/53) (data not shown). Factually, *P. multocida* was not recorded at any examined farm at all neither by conventional methods nor by PCR.

Notably, all infection probabilities were reported in chickens` farms with different production sectors; in broiler chicken farms (n=22), *E. coli* was highly detected (77.27%) followed by *Ps. aeruginosa* (50%), other bacterial infections were also recorded, but with lower percentages. In broiler breeder farms (n=27), a high detection rate was recoded for *Ps. aeruginosa* (66.67%) followed by *M. haemolytica* (59.26%), *E. coli* (37.03%), *M. gallisepticum* (25.93%) and lastly *S. aureus* (11.11%); but, a mixed infection of *E. coli* and *M. gallisepticum* was detected in the layer farm. On the other hand, the ostrich farm was infected by *E. coli* only ,while, no respiratory bacterial pathogens were reported in examined duck farms (n=2) at all (Table 3).

Table 2. Respiratory bacterial pathogens recovered from poultry farms (n=53) by classical culture methods and PCR.

Bacterial pathogen	Classical culture results No. of farms (%)	PCR results No. of farms (%)	Proportion difference (95%CI)	*P* value
E. coli	25 (47.17)	29 (54.71)	7.55% (-1.55 - 7.55%)	0.125
M. gallisepticum	3 (5.66)	11 (20.75)	15.09% (3.94 - 15.09%)	0.008
S. aureus	2 (3.77)	4 (7.55)	3.77% (-2.58 - 3.77%)	0.500
Ps. aeruginosa	8 (15.09)	29 (54.71)	39.62% (26.86 - 39.62%)	< 0.0001
M. haemolytica	11 (20.75)	19 (35.85)	15.09% (3.94 -15.09%)	0.008

CI: Confidence interval. *P* value < 0.05 was considered to be statistically significant.

Table 3. Occurrence of bacterial infections in 53 poultry farms in Egypt.

Bacterial infections	Positive farms (%)	Poultry species (No. of farms)*
E. coli	15 (28.3)	broiler (10), broiler breeder (4), ostrich (1)
M. gallisepticum	1 (1.89)	broiler breeder
Ps. aeruginosa	3 (5.66)	broiler (2), broiler breeder (1)
M. haemolytica	1 (1.89)	broiler breeder
E. coli + *M. gallisepticum*	2 (3.77)	broiler breeder (1), layer (1)
E. coli + *Ps. aerouginosa*	5 (9.4)	broiler (3), broiler breeder (2)
E. coli + *M. haemolytica*	2 (3.77)	broiler breeder
S. aureus + *Ps. aeruginosa*	1 (1.89)	broiler
M. haemolytica + *Ps. aeruginosa*	9 (16.98)	broiler breeder
M. gallisepticum + *Ps. aeruginosa*	2 (3.77)	broiler breeder
M. gallisepticum + *Ps.aeruginosa* + *M. haemolytica*	1 (1.89)	broiler
S. aureus + *Ps. aeruginosa* + *M. haemolytica*	1 (1.89)	broiler breeder
E. coli + *Ps.aeruginosa* +*M. haemolytica*	2 (3.77)	broiler
E. coli + *M. gallisepticum* + *Ps. aeruginosa*	2 (3.77)	broiler
E. coli + *M. gallisepticum* + *Ps. aeuginosa* +*M. haemolytica*	1 (1.89)	broiler breeder
M. gallisepticum + *S. aureus* + *Ps. aeruginosa* + *M. haemolytica*	2 (3.77)	broiler breeder
Negative	3 (5.66)	broiler (1), duck (2)

*Results were adopted according to final multiplex and uniplex PCR systems applied in the study.

Overall, according to PCR results, out of 53 examined farms, 20 (37.73%) were with single bacterial infection, 30 (56.6%) associated with mixed infections, meanwhile, 3 farms only (5.66%) showed negative results. Possible conditions for bacterial infections associated with respiratory signs in poultry are shown in Table (3).

Discussion

The causative agents of respiratory diseases in poultry represent a substantial risk of economic and breeding problems in poultry farms throughout the world. The etiology of respiratory disease is complex, often involving more than one pathogen at the same time (4)**.** Interestingly, the classical microbiological techniques currently in use for bacterial detection and identification are satisfactory in most situations and remain necessary for drug susceptibility testing, but the complexity associated with them makes alternative approaches attractive (21). This is an argument for the utilization of molecular diagnostics relying on the presence of bacterial DNA, and PCR methods are able to detect small amounts of pathogen even dead ones (22)**.** In the current study, we used PCR to determine whether this technique could offer a potential for rapid diagnosis of most common respiratory bacterial infections in poultry.

Out of 53 examined poultry farms, *E. coli* was highly recorded (47.17%) followed by *M. haemolytica* (20.75%), *Ps. aeruginosa* (15.09%) and *M. gallisepticum* (5.66%), while *S. aureus* was detected with a lower percentage (3.77%) by conventional identification-methods. Indeed, *P. multocida* was not recorded at any examined farm at all.

Multiplex PCR assay was optimized for successful detection of four respiratory pathogens including *E. coli, M. gallisepticum*, *S. aureus* and *Ps. aeruginosa* from clinical specimens collected from 53 diseased poultry farms, while *P. multocida* and *M. haemolytica* were detected in uniplex PCR systems . According to PCR results, the most commonly reported bacterial pathogens were *E. coli* and *Ps. aeruginosa* (54.71% each). Both microorganisms were recorded by lower percentages in previous studies as Masdooq *et al.* (23), who recorded *E. coli* in chicken flocks showed respiratory disorders with a percentage of 29% and Slman and Madlool (24), who detected *Ps. aeruginosa* in lung tissues of poultry with an incidence of 35%. Possible explanations for this variation may include different geographical areas and different laboratory technique used in microbiological diagnosis of respiratory infections in poultry.

In particular, the PCR assays showed a significantly higher rate of detection of respiratory bacterial pathogens in poultry farms than that was detected by the conventional culture methods (Table 3) and these findings were commonly reported by other investigators (25)**.**

In conclusion, single-tube multiplex PCR holds potential to be more reliable, economical and rapid diagnostic tool for simultaneous detection of avian respiratory bacteria in clinical samples of poultry and provides an efficient way to ask several related epidemiological questions.

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