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Genetic diversity of different avian strains of *Pasteurella multocida* recovered during four outbreaks in India

Kumaragurubaran KARTHIK*, Vishwanathan DILLIBABU, Ramalingam MAHAPRABHU, Ramasamy BHARATHI, Kaliyaperumal MANIMARAN, Kulasekaran SHOBA
Central University Laboratory, Tamil Nadu Veterinary and Animal Sciences University,
Madhavaram Milk Colony, Chennai, Tamil Nadu, India

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Abstract: *Pasteurella multocida* causes fowl cholera in avian species and type A is predominantly reported from outbreaks of fowl cholera. Biochemical, serological, and molecular methods are employed for its diagnosis and typing. Utilizing rapid molecular tools like repetitive extragenic palindromic (REP) PCR and enterobacterial repetitive intergenic consensus (ERIC) PCR, *P. multocida* isolates from four outbreaks (three from Chennai and one from Ahmadabad, India) were characterized and typed to determine their relationships. A total of 36 isolates were recovered from the outbreaks, including one isolate from a parakeet, which was also subjected to characterization by conventional and molecular analysis. All of the isolates were found to be capsular type A based on PCR assay capsular typing. ERIC- and REP-PCR showed differences in the banding patterns among different outbreak isolates, and also among the geographical regions. Differences were also noticed among different host species, as the banding pattern in the ERIC- and REP-PCR differed; the analysis of results also revealed the same. All of the isolates were found to be sensitive to enrofloxacin and cefotaxime among the antibiotics used in the study. It was found that different strains might have been involved in the different outbreaks reported in the study. The results show that molecular typing methods like ERIC- and REP-PCR are useful epidemiological tools for classifying the strains.

Key words: *Pasteurella multocida*, ERIC-PCR, REP-PCR, fowl cholera, genotyping

1. Introduction

Fowl cholera (FC) is an acute septicemic deadly disease of various poultry species caused by *Pasteurella multocida*, a gram-negative, nonmotile, nonspore-forming, aerobic, rod-shaped bacterium (1). *Pasteurella multocida* mainly colonizes the respiratory tract of healthy animals and birds, but can cause disease under stress conditions (2). FC has been reported throughout the world and is known to cause significant mortality, leading to economic losses for poultry farmers (3,4). FC outbreaks have been reported from several parts of India at various times (5). Based on the sugar (dulcitol and sorbitol) fermentation test, *P. multocida* is divided into 3 subspecies, namely *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida*, and *P. multocida* subsp. *septica* (1). Diagnosis of FC relies on isolation and identification of the causative agent by employing conventional methods, followed by capsular typing using an indirect hemagglutination test and serotyping (6,7). Though 4 serogroups (A, B, D, and F) have been reported from poultry, *Pasteurella multocida* type A:1 has been implicated in the majority of outbreaks of FC (8). Conventional diagnosis and phenotypic differentiation tools have long been used to identify FC isolates, but these methods are time-consuming and laborious; hence, molecular assays like polymerase chain reaction (PCR) are employed for accurate diagnosis (9,10). Recent advances in diagnosis have aided in molecular characterization of the isolates using techniques like enterobacterial repetitive intergenic consensus (ERIC) PCR, restriction endonuclease analysis (REA), random amplified polymorphic DNA (RAPD), multilocus sequencing typing (MLST), and repetitive extragenic palindromic (REP) PCR (4,11,12). These methods have higher reproducibility and better discriminatory power; thus, these methods can be employed for epidemiological studies of the isolates (12).

A few studies have reported the isolation of similar strains of *P. multocida* from the same farm over time, while some studies have reported the isolation of different genotypes during different outbreaks over time from the same farm (13,14). ERIC-PCR and REP-PCR were used in earlier studies to characterize the avian *P. multocida* isolates recovered from different regions of India to

* Correspondence: karthik_2bvsc@yahoo.co.in

determine their genetic diversity (8). Four FC outbreaks were investigated during the period of one year (June 2016 to June 2017); a study was conducted to characterize the isolates to determine their relatedness. Hence, the present study was undertaken to characterize the *P. multocida* isolates using molecular tools to determine their diversity in different avian species among the same and different outbreaks.

2. Materials and methods

2.1. Sample collection, bacterial isolation, and identification

From the period of June 2016 to June 2017, four outbreaks (three from Chennai, Tamil Nadu State, India, and one from Ahmadabad, Gujarat State, India) of FC were reported. There was more than 60% mortality in all of the outbreaks; dead birds were subjected to postmortem examination. Heart blood swab, liver, and long bones were collected for isolation and identification of the causative agent. Samples were processed at Central University Laboratory of Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai, India. All of the samples were processed on blood agar, brain-heart infusion agar, and MacConkey agar. Isolated colonies were subjected to Gram staining and biochemical tests like catalase, oxidase, indole, methyl red, Voges–Proskauer, citrate, and urease tests. During the same time period, an isolate of *P. multocida* was also recovered from a parakeet that was brought to the Madras Veterinary College Clinics of TANUVAS. Based on conventional tests, a total of 36 isolates were isolated, and the details of the isolates are shown in Table 1.

2.2. Antimicrobial susceptibility testing

All the isolates were tested with 7 commonly used antimicrobial disks, namely amoxyclav, cefotaxime, gentamicin, tetracycline, norfloxacin, enrofloxacin, and doxycycline, to find the sensitivity pattern using the Kirby–Bauer disk diffusion method (15).

2.3. DNA extraction and capsular typing PCR

Briefly, 1 mL of overnight culture of each isolate in BHI broth was pelleted and used for DNA extraction using a DNeasy Blood and Tissue Kit (QIAGEN, Germany) following the manufacturer's instructions. PCR assay was carried out for confirmation of the isolate as *P. multocida*, targeting the *kmt*1 gene by employing the established primers of Townsend et al. (16). All of the DNA was later subjected to a multiplex PCR assay using five primer sets as per the method described by Townsend et al. (17), so as to type isolates based on their capsules. PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. Agarose gel (1.5%) electrophoresis was carried out to separate the bands and was visualized under the Gel Doc XR$^+$ system (Bio-Rad, United States).

2.4. Enterobacterial repetitive intergenic consensus (ERIC) PCR

ERIC-PCR targeting the palindromic sequences was carried out using the established primers (ERIC1R 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2 5'-AAGTAAGTGACTGGGGTGAGCG-3') and the PCR conditions of Versalovic et al. (18). PCR conditions employed were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Agarose gel electrophoresis was carried out to verify the presence or absence of polymorphic fragments using the banding pattern.

2.5. Repetitive extragenic palindromic (REP) PCR

REP-PCR was also performed for the entire extracted DNA, employing established primers (REP1R-1Dt 3'-CGGNCTCANGCNGCNNNN-5' and REP2-1Dt 5'-NCGNCTTATCNGGCCTAC-3') and the PCR conditions of Versalovic et al. (18). The PCR conditions employed were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 42 °C for 1 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Agarose gel electrophoresis was carried out to separate the bands and was visualized under the Gel Doc XR$^+$ system.

2.6. Analysis of ERIC- and REP-PCR results

ERIC-PCR and REP-PCR profiles were analyzed using GelCompar II v. 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium). Dice's similarity coefficient was used to cluster the isolates, and the unweighted pair group method with arithmetic mean (UPGMA) was used to analyze and establish relationships among isolates. A similarity genetic index of 90% was selected arbitrarily to determine the groups. The discriminatory ability of the two typing methods was determined by application of Simpson's index of diversity (19).

3. Results

3.1. Bacterial isolation and identification

Colonies having characteristics of dewdrop and nonhemolytic appearance on blood agar were used further for biochemical testing. Upon Gram staining, the appearance of gram-negative coccobacilli was noticed. Isolated colonies showed no growth on MacConkey agar, and tests like catalase, oxidase, and indole were positive for all of the isolates. Methyl red, Voges–Proskauer,

Table 1. List of isolates recovered during the outbreaks.

S. no.	Farm no.	Place	State	Host	Year	Isolate no.
1	1	Chennai	Tamil Nadu	Chicken	2016	IN/PM/Chicken/Chennai/Farm1/Isolate 1/2016
2						IN/PM/Chicken/Chennai/Farm1/Isolate 2/2016
3						IN/PM/Chicken/Chennai/Farm1/Isolate 3/2016
4						IN/PM/Chicken/Chennai/Farm1/Isolate 4/2016
5						IN/PM/Chicken/Chennai/Farm1/Isolate 5/2016
6						IN/PM/Chicken/Chennai/Farm1/Isolate 6/2016
7						IN/PM/Chicken/Chennai/Farm1/Isolate 7/2016
8						IN/PM/Chicken/Chennai/Farm1/Isolate 8/2016
9						IN/PM/Chicken/Chennai/Farm1/Isolate 9/2016
10						IN/PM/Chicken/Chennai/Farm1/Isolate 10/2016
11						IN/PM/Chicken/Chennai/Farm1/Isolate 11/2016
12						IN/PM/Chicken/Chennai/Farm1/Isolate 12/2016
13	2	Chennai	Tamil Nadu	Chicken	2016	IN/PM/Chicken/Chennai/Farm 2/Isolate 1/2016
14						IN/PM/Chicken/Chennai/Farm 2/Isolate 2/2016
15						IN/PM/Chicken/Chennai/Farm 2/Isolate 3/2016
16						IN/PM/Chicken/Chennai/Farm 2/Isolate 4/2016
17	2	Chennai	Tamil Nadu	Chicken	2017	IN/PM/Chicken/Chennai/Farm 2/Isolate 1/2017
18						IN/PM/Chicken/Chennai/Farm 2/Isolate 2/2017
19						IN/PM/Chicken/Chennai/Farm 2/Isolate 3/2017
20						IN/PM/Chicken/Chennai/Farm 2/Isolate 4/2017
21						IN/PM/Chicken/Chennai/Farm 2/Isolate 5/2017
22						IN/PM/Chicken/Chennai/Farm 2/Isolate 6/2017
23						IN/PM/Chicken/Chennai/Farm 2/Isolate 7/2017
24	2	Chennai	Tamil Nadu	Duck	2017	IN/PM/Duck/Chennai/Farm 2/Isolate 1/2017
25						IN/PM/Duck/Chennai/Farm 2/Isolate 2/2017
26						IN/PM/Duck/Chennai/Farm 2/Isolate 3/2017
27						IN/PM/Duck/Chennai/Farm 2/Isolate 4/2017
28						IN/PM/Duck/Chennai/Farm 2/Isolate 5/2017
29						IN/PM/Duck/Chennai/Farm 2/Isolate 6/2017
30						IN/PM/Duck/Chennai/Farm 2/Isolate 7/2017
31						IN/PM/Duck/Chennai/Farm 2/Isolate 8/2017
32	1	Ahmadabad	Gujarat	Chicken	2017	IN/PM/Chicken/Ahmadabad/Farm 1/Isolate 1/2017
33						IN/PM/Chicken/Ahmadabad/Farm 1/Isolate 2/2017
34						IN/PM/Chicken/Ahmadabad/Farm 1/Isolate 3/2017
35						IN/PM/Chicken/Ahmadabad/Farm 1/Isolate 4/2017
36	-	Chennai	Tamil Nadu	Parakeet	2017	IN/PM/Parakeet/Chennai/Isolate 1/2017

citrate, and urease tests were negative for the isolates. A total of 36 isolates were recovered and the details are shown in Table 1. Among the isolates, 4 isolates were from Ahmadabad, Gujarat, and the remaining 32 isolates were from Chennai, Tamil Nadu. Host-wise, the classification shows that 8 isolates are from ducks, 1 isolate is from a parakeet, and the remaining 27 isolates are from chickens.

3.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of the different isolates are shown in Table 2. All of the isolates were sensitive to cefotaxime and enrofloxacin. Other antimicrobials—amoxyclav, gentamicin, tetracycline, norfloxacin, and doxycycline—had 55.56%, 72.22%, 77.78%, 47.22%, and 61.11% sensitivity, respectively.

Table 2. Antibiotic sensitivity pattern of the *P. multocida* isolates.

S. No.	Antimicrobial agent	Concentration	No. of isolates (percentage of isolates)		
			Sensitive	Intermediate sensitive	Resistant
1	Amoxyclav	30 µg	20 (55.56%)	12 (33.33%)	4 (11.11%)
2	Cefotaxime	30 µg	36 (100%)	-	-
3	Gentamicin	10 µg	26 (72.22%)	10 (27.78%)	-
4	Tetracycline	30 µg	28 (77.78%)	-	8 (22.22%)
5	Norfloxacin	20 µg	17 (47.22%)	11 (30.56%)	8 (22.22%)
6	Doxycycline	10 µg	22 (61.11%)	12 (33.33%)	2 (5.56%)
7	Enrofloxacin	10 µg	36 (100%)	-	-

3.3. Capsular typing PCR

PCR targeting the *kmt*1 gene showed a band at ~460 bp, which was the expected size (Figure 1), and capsular typing results showed that all the isolates were of type A, as the gel electrophoresis band corresponded to ~1044 bp (Figure 2).

3.4. Enterobacterial repetitive intergenic consensus (ERIC) PCR

ERIC-PCR of all the avian isolates had amplified products ranging from ~200 bp to ~3000 bp. Only 10 prominent bands were selected for UPGMA analysis, although there were minor faint bands. At a 90% cutoff value, 10 different profiles were recognized; isolates could be grouped into 9 miniclusters having 2, 4, and 8 isolates among them (Figure 3). The index of discrimination (D value) was found to be 0.88. Duck isolates of the year 2017 from Farm 2 in Chennai clustered together while chicken isolates of the year 2017, also from Farm 2 in Chennai, showed 2 miniclusters and 1 unique pattern. Chicken isolates from the Ahmadabad region had 2 miniclusters, while chicken isolates from Farm 1 had one major cluster and one minor cluster. Chicken isolates from the year 2016 from Farm 2 in Chennai formed one major cluster. The parakeet isolate was unique and did not form a cluster with other isolates.

3.5. Repetitive extragenic palindromic (REP) PCR

REP-PCR isolates had banding patterns ranging from ~200 bp to ~3000 bp. Only 14 prominent bands were selected for UPGMA analysis, although there were minor faint bands. At a 90% cutoff value 8 different profiles were recognized, and isolates could be grouped into 6 miniclusters having 2, 4, 6, and 12 isolates among them (Figure 4). The index of discrimination was found to be 0.83. All of the duck isolates from the year 2017 from Farm 2 clustered together, while chicken isolates from the year 2017 from the same farm formed one major cluster and one separate isolate. Chicken isolates from Farm 2 (2016) clustered together, while Ahmadabad isolates had one minor cluster and one separate isolate. Similarly, all chicken isolates from Farm 1 in Chennai clustered together, while the parakeet isolate had a unique pattern.

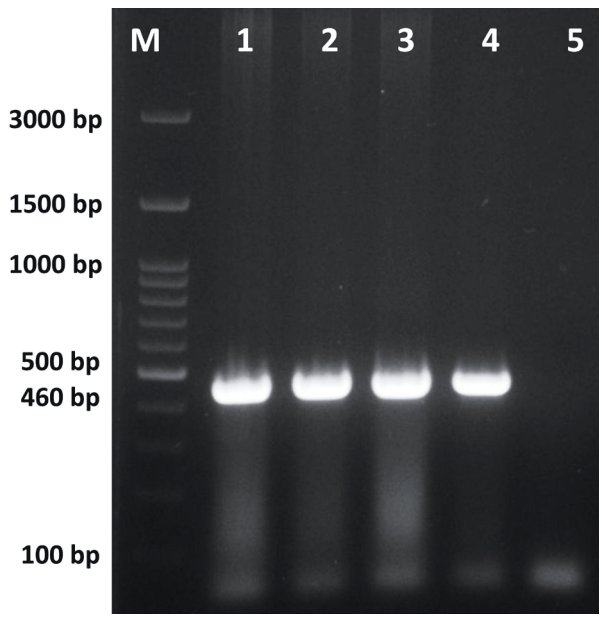

Figure 1. Gel electrophoresis image of *P. multocida* specific PCR. M: 100-bp DNA ladder; Lane 1: IN/PM/Duck/Chennai/Farm 2/Isolate 1/2017; Lane 2: IN/PM/Chicken/Chennai/Farm 2/Isolate 1/2016; Lane 3: IN/PM/Chicken/Ahmadabad/Farm 1/Isolate 1/2017; Lane 4: IN/PM/Parakeet/Chennai/Isolate 1/2017; Lane 5: Negative control.

4. Discussion

The occurrence of fowl cholera has been reported from various parts of India and worldwide, even though vaccination has been performed in many places (20,21). Conventional bacteriological examinations have been employed for diagnosis of the disease, while serological

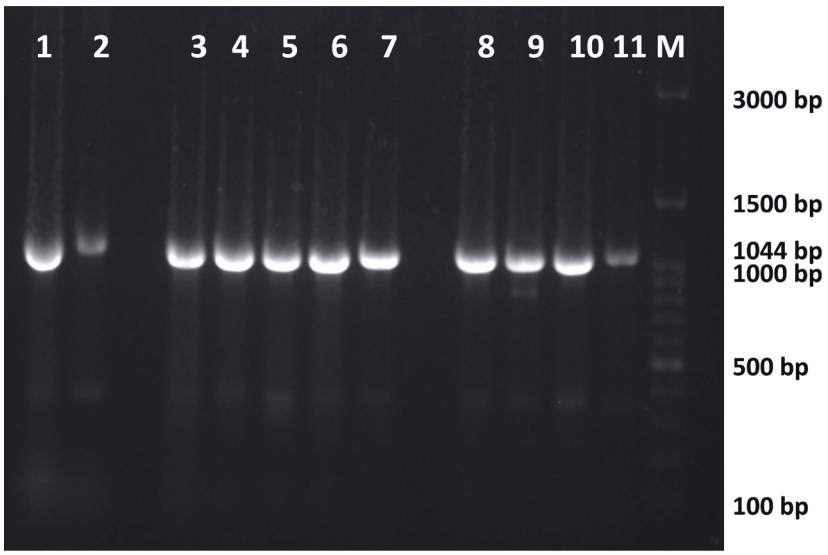

Figure 2. Gel electrophoresis image of capsular typing PCR. M: 100-bp DNA ladder; Lane 1: IN/PM/Chicken/Chennai/Farm1/Isolate 1/2016; Lane 2: IN/PM/Chicken/Chennai/Farm1/Isolate 2/2016; Lane 3: IN/PM/Chicken/Chennai/Farm1/Isolate 5/2016; Lane 4: IN/PM/Chicken/Chennai/Farm1/Isolate 10/2016; Lane 5: IN/PM/Chicken/Chennai/Farm 2/Isolate 1/2016; Lane 6: IN/PM/Chicken/Chennai/Farm 2/Isolate 1/2017; Lane 8: IN/PM/Chicken/Chennai/Farm 2/Isolate 1/2017; Lane 9: IN/PM/Duck/Chennai/Farm 2/Isolate 1/2017; Lane 10: IN/PM/Chicken/Ahmadabad/Farm 1/Isolate 1/2017; Lane 11: IN/PM/Parakeet/Chennai/Isolate 1/2017.

methods have been used for typing the isolates. These methods have their own drawbacks, as conventional biochemical tests are time-consuming, and serotyping is also laborious and time-consuming (9,10). Hence, outbreaks involving different strains require exhaustive investigation in order to type the isolates. Thus, in the present study, along with conventional bacteriological tests for identification of the pathogen, molecular assays were used to type the isolates based on their capsule. ERIC- and REP-PCR were also used to group the isolates based on their banding pattern to establish the relationships among the isolates.

In the present study, *P. multocida* was isolated from 4 separate outbreaks (3 from Chennai and 1 from Ahmadabad). Two outbreaks were reported from two different farms (1 and 2) in Chennai during the year 2016. In 2017, Farm 2 of Chennai had another outbreak where chicken and ducks were affected, whereas only chickens were affected in an outbreak in Ahmadabad. Earlier studies also reported an outbreak of FC in ducks and chickens from the same area (22). Susceptibility of birds to FC varies among avian species and age groups (23).

A total of 36 isolates were recovered during the study, and the agent was confirmed as *P. multocida* by bacteriological examination. Due to the higher sensitivity of molecular tests, PCR-based typing methods were used in the study to type the isolates based on their capsules. All of the isolates in the present study were found to be capsular type A. It was reported that *P. multocida* capsular type A is predominantly prevalent among Indian poultry species and causes an acute form of FC (8,24). An earlier investigation showed that out of the 123 isolates, 20 isolates could not be typed by conventional methods, while molecular typing enabled their classification (25). Similarly, it was found that repeated subculture may lead to loss of the capsule; hence, serotyping becomes difficult (26). Serogroups A, D, and F failed to agglutinate homologous antisera, leading to less sensitivity of serotyping (27). The difficulty in raising or obtaining capsular-specific antisera and the disadvantages of serotyping justify the shift towards molecular typing methods. Hence, molecular assay was carried out in the present study to identify the capsular type of the isolates.

ABST results show that enrofloxacin and cefotaxime had 100% sensitivity against all of the isolates, which was in accordance with earlier studies conducted by Sellyei et al. (7), where enrofloxacin had 100% sensitivity against poultry isolates. Similarly, cefquinome, another member of the cephalosporin group, had 100% sensitivity (7). There was no single antimicrobial that was found to be 100% resistant against all of the isolates, which was in agreement with the findings of Rigobelo et al. (28). Although antimicrobial resistance development has

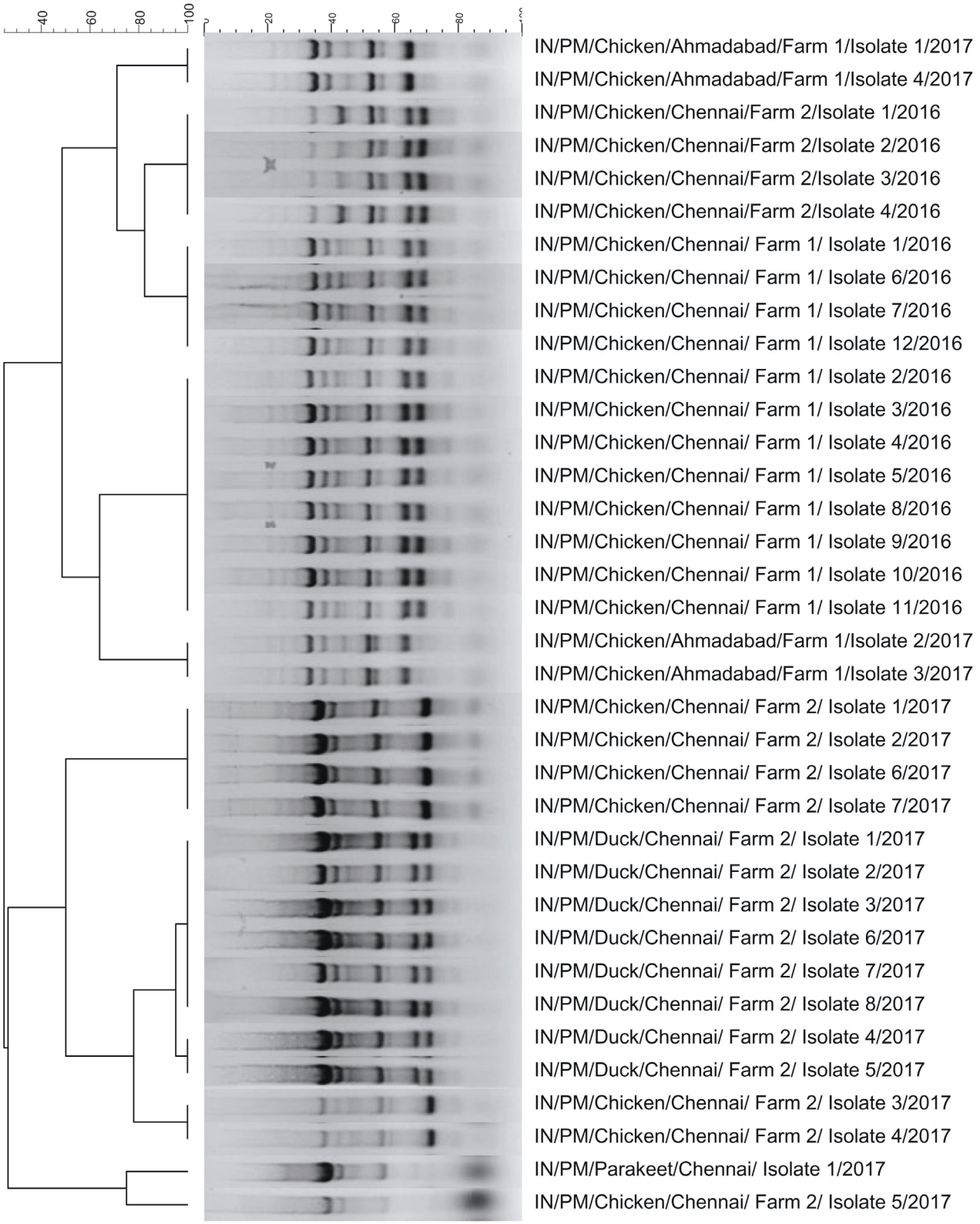

Figure 3. Dendrogram showing the cluster analysis of ERIC-PCR profiles of *P. multocida* strains.

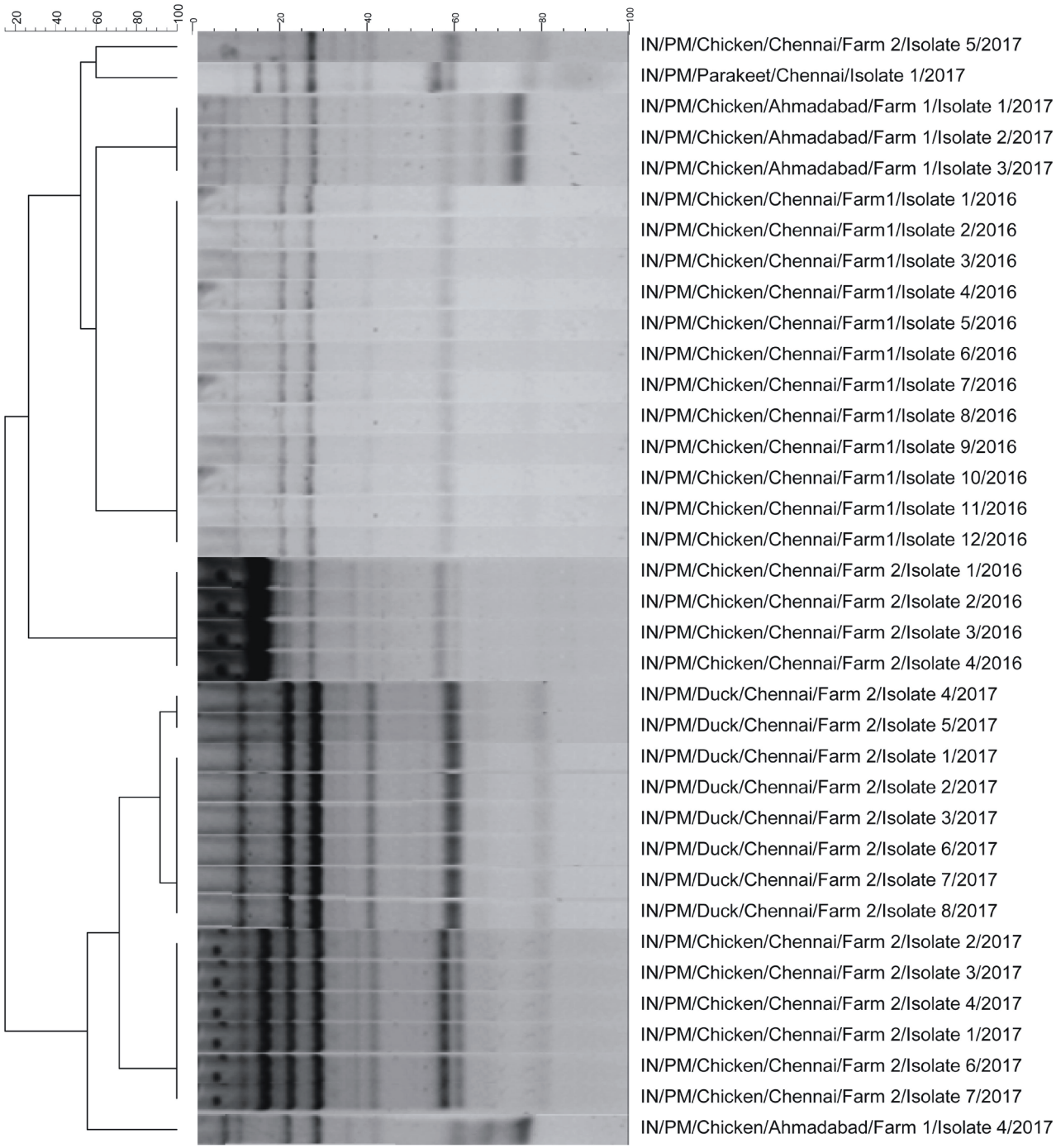

Figure 4. Dendrogram showing the cluster analysis of REP-PCR profiles of *P. multocida*. strains.

increased in recent years in several bacteria including *P. multocida*, some isolates in the study showed sensitivity towards all of the antimicrobials used.

Basic bacteriological tests and capsular typing by PCR did not allow differentiation of isolates from 4 different outbreaks (chickens and ducks) or from the parakeet. Hence, to distinguish among the isolates and between the outbreaks, ERIC- and REP-PCR were employed, which allowed clustering of the isolates. Farm 2 had 2 outbreaks during the period of study, and it was speculated that the isolates would be similar, but ERIC- and REP-PCR analysis showed that the 2016 and 2017 chicken outbreaks formed different clusters. In 2017, the outbreak in Farm 2 started initially in ducks, immediately followed by

chickens, but analysis showed that the isolates were not the same. However, they were clustering together at 90% similarity level. Based on the banding pattern of the isolates in ERIC- and REP-PCR, analysis was carried out using GelCompar II software. Our results were similar to those of earlier studies that demonstrated that multiple strains can be identified from the same outbreak (4,29). Farm 2 is a multispecies farm where different species of poultry, namely chickens, ducks, turkeys, guinea fowl, and pigeons, are reared, along with other animal species like cattle, sheep, goats, and pigs. Ducks had been recently purchased from another farm and were reported to have FC; this could be the reason for the difference in the isolates between ducks and chickens on the same farm. Similarly, the 2016 and 2017 outbreaks from Farm 2 were also found to be different. Olson and Wilson (30) also reported multiple strain involvement at the same turkey farm at different time points. Shivachandra et al. (4) suggested that high flock density may lead to mutation of the isolates, causing repeated outbreaks. Although Farm 1 is located within a 20-km range of Farm 2, analyses showed that the isolates from the outbreaks were different, which was similar to results of the study conducted by Blackall et al. (31). The banding patterns obtained for isolates from Ahmadabad and Chennai were different in both PCR assays, indicating that the isolates differ between the regions. Similarly, pattern differences were noticed between ducks, chickens, and the parakeet. There exist differences at the genetic level of the isolates obtained in the study; this merits further study in order to draft a better vaccine to prevent and control this economically important disease of poultry.

There are several molecular typing tools available, and several tools have been used for typing *P. multocida* isolates earlier. REA using *Hpa*II, ERIC-PCR, and REP-PCR were reported to have higher discriminatory potential to differentiate the isolates (32). Other methods like pulse field gel electrophoresis, amplified fragment length polymorphism, and MLST were found to be more sensitive assays than ERIC- and REP-PCR. However, these assays are complex, stringent, and laborious while ERIC- and REP-PCR are easy to perform and rapid, and analysis of the banding pattern is also easier (4,33). Shivachandra et al. (4) also stated that REA was not useful for differentiating isolates within the same region or the same outbreak. Since outbreaks from the same region as well as from distinct regions are involved in this study, ERIC- and REP-PCR were carried out. ERIC-PCR had a higher D value than REP-PCR (0.88 vs. 0.83), which was similar to the results of Shivachandra et al. (4) Still, both were effective in discriminating the isolates easily without stringent analysis. Thus, to discriminate the isolates from outbreaks of different regions or the same region and time frame, ERIC- and REP-PCR can be employed with ease.

Thus, the current report shows that FC still prevails in several parts of India; there is a need for exhaustive epidemiological study and typing studies to identify the strains that cause outbreaks. Molecular typing studies and fingerprinting techniques should be carried out to identify the strains involved in outbreaks, as conventional phenotypic or serotypic classification is not sensitive enough or feasible. Clear knowledge on the involvement of particular strains can aid in designing better prevention and control strategies by developing vaccines employing native strains involved in outbreaks of FC.

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