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Identification and Antibiotic Resistance of *Pasteurella multocida* Isolated from Infected Layer Chickens in West Java, Indonesia

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ABSTRACT

Bacterial infections, such as those caused by *Pasteurella multocida* serotype A, pose significant threats to poultry farming. The use of antibiotics to treat these infections can lead to antibiotic resistance. The present study aimed to identify *Pasteurella multocida* from 14 Hisex Brown layer chicken hen farms, with chikens aged 25-55 weeks, in West Java, Indonesia, and to evaluate their resistance to various antibiotics. Three samples from each farm were collected from dead chickens having symptoms of fowl cholera. Initially, the study involved isolating and identifying isolates from liver, heart, and lung organs via polymerase chain reaction. The colony was then tested for antibiotic resistance using the disk diffusion method. The results showed that 13 samples were *Pasteurella multocida* and nine were serotype A. The test results also indicated that all isolates were resistant to colistin (10 µg) and sensitive to tetracycline (30 µg), amoxicillin (25 µg), enrofloxacin (5 µg), sulfamethoxazole (25 µg), lincomycin (109 µg), and ciprofloxacin (5 µg). The study concluded that none of the *Pasteurella multocida* type A isolates were any longer sensitive to colistin, with some isolates still sensitive to tetracycline, amoxicillin, enrofloxacin, sulfamethoxazole, lincomycin, and ciprofloxacin, and two isolates showing multidrug resistance patterns.

Keywords: Antibiotic, Fowl cholera, *Pasteurella multocida*, Layer chicken

INTRODUCTION

Animal protein is an essential nutritional requirement of humans, and poultry is one of the most affordable sources of this protein. As such, the availability of poultry products needs to be increased to meet the growing demand (Choi et al., 2023). However, the poultry farming sector faces several challenges, including the threat of avian cholera. Avian cholera, also known as fowl cholera, is a poultry disease caused by a contagious bacterial infection that is widespread worldwide (Singh et al., 2014). This disease is caused by infection with the bacterium *Pasteurella multocida* (Mohamed and Mageed, 2014).

Pasteurella multocida (*P. multocida)* is a bacterium that can survive with or without oxygen and is classified under Gram-negative bacteria. while *P. multocida* exhibits robust growth on blood and chocolate agar, it fails to cultivate on MacConkey agar, Eosin Methylene Blue (EMB) agar, or other selective differential media. *P. multocida* is classified into five serogroups based on its capsule type, namely A, B, D, E, and F, and sixteen serotypes ranging from serotypes 1 to 16. Serotypes of *P. multocida* with capsules exhibit higher virulence as compared to non-capsulated serotypes. Diseases resulting from this infection can be caused by several serotypes, such as capsule type A (Harper et al., 2006). Among various *P. multocida* serotypes, serotype A is most frequently associated with fowl cholera Serotype (Wilkie et al., 2012). Infections with *P. multocida* may cause pathological lesions in several organs including the heart, intestines, kidneys, and liver, which are often characterized by petechiae and white spots (Zainuddin, 2008).

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Fowl cholera affects not only poultry livestock but also pet birds, turkeys, and ducks. Birds affected by cholera have shown two types of symptoms, including acute and chronic. Acute cholera symptoms, including fever, anorexia, mucus discharge from the beak, diarrhea, cyanosis, and increased respiratory rate, occur shortly before the death of the bird. In contrast, chronic symptoms may occur after the acute phase (Blakey et al., 2019). Fowl cholera is commonly managed by administering broad-spectrum antibiotic preparations mixed into the birds' feed and drinking water (Gray et al., 2021). Most antibiotics are used for infected cases of avian cholera. Inappropriate long-term antibiotic use can lead to antibiotic resistance. Antibiotic resistance to pathogenic bacteria in livestock is a significant health concern that needs attention (Dashe et al., 2013). Some antibiotics that should be considered in antibiotic resistance testing include penicillin, β-lactam/β-lactamase inhibitor, cephalosporin, fluoroquinolone, tetracycline, and macrolide groups (Kapoor et al., 2017). The several antibiotics used for pasteurellosis therapy have exihibited varying degrees of effectiveness and sensitivity. Aminoglycoside antibiotics, *in vitro*, are the least effective against *P. multocida* (Hurtado et al., 2020). Additionally, vancomycin and clindamycin are resistant to *P. multocida*. However, *P. multocida* is highly susceptible to fluoroquinolone and oxazolidinone groups. Isolates of *P. multocida* from animals also show resistance to tetracycline. Currently, penicillin and expanded-spectrum cephalosporin are the preferred antibiotics for treating infections by *P. multocida* (Huang et al., 2009; Hurtado et al., 2020).

The bacterium responsible for avian cholera can bring about substantial financial losses in the poultry industry. Hence, identifying and characterizing *P. multocida* bacteria are essential steps for accurate poultry therapy. It is also essential to assess the resistance profile of each tested isolate in order to establish the efficacy of antibiotics in treating this bacterial infection.

In this line, the present study aimed to identify *P. multocida* bacteria and characterize the antibiotic susceptibility profile from cases of fowl cholera in layer chickens.

MATERIALS AND METHODS

Ethical approval

Ethical approval for this study was obtained from the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Science, IPB University, Indonesia, under the approval number 121/SKE/X/2023.

Samples

The samples were collected between 2016 and 2020 from 14 suspected *P. multocida* archives isolated from Hisex Brown layer chicken farms in Sukabumi, West Java, where the chickens were aged 25-55 weeks. The cases involved chickens exhibiting symptoms of fowl cholera, such as cyanosis, fever, mucous discharge, diarrhea, and sudden deaths. These were the cases with a high mortality rate. Archive isolates were obtained from liver, heart, and lung organs showing abnormalities or lesions combined based on the original sample pens. The organs were washed with sterile phosphate buffer saline (PBS) at pH 7.4, and swab samples were collected from the inner parts using sterile cotton swabs. The swabs were then mixed with 2 mL of sterile PBS and cultured on blood agar (Oxoid, UK). The colonies grown on blood agar were observed macroscopically, and Gram staining was performed to observe bacterial morphology microscopically, confirming the presence of bipolar Gram-negative coccoid-shaped bacteria (Desem et al., 2023). The suspected isolates were subsequently stored in freeze-dried ampoules.

Culture, identification, and confirmation of *Pasteurella multocida*

To culture the bacteria, 300 µL of Brain Heart Infusion (BHI) broth media was mixed with *P. multocida* from each freeze-dried ampoule of archive isolates and homogenized by shaking. The homogenized solution was then inoculated on the edge of blood agar and MacConkey agar media (Oxoid, UK), streaked, and incubated at 37°C for 18-24 hours (Desem et al., 2023). The grown colonies were observed both macroscopically and microscopically using Gram staining to determine purity. Macroscopically, *P. multocida* colonies appear round, shiny, and whitish-grey, with varied sizes. Microscopically, however, The isolates exhibit a characteristic bipolar coccoid morphology and are Gram-negative. Pure colonies obtained from each isolate were further subjected to biochemical tests, such as catalase, oxidase, indole tests, and molecular confirmation through polymerase chain reaction (PCR, Nugroho et al., 2022).

Phenotypic colony identification

The bacterial identification method followed the protocol outlined by Nugroho et al. (2022). Pure colonies from each sample were subjected to the catalase test. The colonies were taken and mixed with 200 µL of 3% H_2O_2 on a glass slide; the formation of gas bubbles indicated a

positive result. Meanwhile, the oxidase test was conducted by adding a single colony loop needle to an oxidase paper; a color change of the paper to purple indicated a positive result. The indole test involved adding Kovacs reagent to the media inoculated with *P. multocida* bacteria; the formation of a red ring on the top of the growth media showed a positive result.

Total bacterial DNA extraction

The DNA extraction process was conducted to get genetic material from *P. multocida* cell samples previously used for testing. The boiling method was employed for DNA extraction, in which several bacterial single colonies from a blood agar culture were combined with 1 mL of PBS in a 1.5 mL microtube. The mixture was then homogenized using a vortex. The suspension was then centrifuged at 10,000 rpm for 10 minutes to pellet the bacterial cells. Subsequently, 100 µL of the pellet was taken and placed into a 1.5 mL microtube. Then, 200 µL of nuclease-free water was added and homogenized using a vortex, and finally incubated at 95 ℃ for 10 minutes. The mixture was centrifuged again at 10,000 rpm, and a 100 microliter portion of the supernatant containing the extracted DNA was collected for potential PCR analysis.

Detection of capA gene specific to *Pasteurella multocida* Serotype A

To detect the capA gene specific to *Pasteurella multocida* Serotype A, a total of 5 µl of extracted DNA was mixed with 25 µl of MyTaq™ HS Red Mix master mix, which included 2 µl of capA forward primer (5′-TGCCAAAATCGCAGTGAG-3′), 2 µl of capA reverse primer (5′-TTGCCATCATTGTCAGTG-3′) with an amplification size of 1044 bp (Townsend et al., 2001; Nugroho et al., 2022), and 16 µl of nuclease-free water. The master mix and the mixture of bacterial DNA extract were then placed into a thermal cycler for DNA amplification. The PCR process was run for 30 cycles. The PCR condition process involved a denaturation step at 95°C for 15 seconds, an annealing step at 55°C for 15 seconds, and an extension step at 72°C for 10 seconds. The PCR product was subsequently analyzed using gel electrophoresis. The amplified samples were observed by electrophoresis, utilizing a 1.5% agarose gel, and stained at a concentration of 0.5 µg/ml ethidium bromide (EtBr). A 100 base pair marker (VC 100 base pair Plus DNA Ladder Vivantis) was employed as a reference for size determination. The electrophoresis procedure was conducted for 35 minutes at a voltage of 80V.

Antibiotic resistance test

The antibiotic resistance test was conducted using the disk diffusion Kirby Bauer method. Mueller Hinton Agar (MHA; Himedia, India) was the media utilized for this assay. Prior to inoculation with bacterial colonies, the agar media was incubated at 37°C for 10-20 minutes. A suspension was prepared from bacterial isolates on Trypticase Soy Agar media (Oxoid, UK) diluted with physiological NaCl and homogenized with a vortex mixer. Turbidity levels were compared with the McFarland 1 standard. A 100 µl suspension was taken and dropped onto MHA media, spread evenly with a sterile cotton bud, and left for 10 minutes (Cappuccino and Welsh, 2018). Antibiotic discs (Oxoid, UK) each containing 25 µg amoxicillin, 30 µg tetracycline, 5 µg ciprofloxacin, 5 µg enrofloxacin, 10 µg colistin, 109 µg lincomycin, and 25 µg sulfamethoxazole were placed on the media inoculated with bacteria, ensuring a minimum distance of 24 mm between the discs. The media was then incubated at 35°C ± 2°C for 18-24 hours (Hudzicki, 2009). After incubation, the diameters of the inhibition zones were measured using a caliper or ruler with a millimeter scale. The results of the antibiotic sensitivity testing were compared with standard inhibition zone diameter values for antibiotics, as outlined in Table 1.

Table 1. The antibiotic resistance parameters in *Pasteurella multocida* from Hisex Brown layer chickens

Group of antibiotics	Antibiotics	Dose	Inhibition zone diameter (mm)			Reference
			Sensitive	Intermediate	Resistance	
Penicillins	Amoxicillin	20/10 µg	≥ 27	–	–	CLSI M45 (2015)
Tetracyclines	Tetracycline	30 µg	≥ 24	–	≤ 24	CLSI M45 (2015)
Fluoroquinolones	Ciprofloxacin	5 µg	≥ 27	–	≤ 27	EUCAST (2024)
	Enrofloxacin	5 µg	≥ 21	17–20	≤ 16	CLSI VET 01S (2015)
Polymyxins	Colistin	5 µg	≥ 17	–	≤ 11	CLSI M45 (2015)
Sulfonamides	Sulfamethoxazole	1,25/ 23,75 µg	≥ 24	–	–	CLSI M45 (2015)

RESULTS

Culture, identification, and confirmation results of *P. multocida*

The results of culture, identification, and confirmation of 14 isolates are detailed in Table 2. Out of 14 archive isolates grown on blood agar media, 13 exhibited colony characteristics with varied sizes, accompanied by round, shiny, and whitish-grey colony formations (Figure 1A). In contrast, no colony growth was observed on MacConkey agar media (Figure 1B). Microscopic examination of all the 13 archive isolates showed conformity with the characteristic features of *P. multocida* bacterial cells, namely a bipolar coccoid shape and a Gram-negative nature (Figure 1C).

The same results were also obtained in the oxidase, catalase, and indole tests for all archive isolates. The 13 isolates exhibited characteristics typical of *P. multocida*. Specifically, pure colonies from each isolate produced gas bubbles upon the addition of H_2O_2 in the catalase test (Figure 1D). They resulted in a color change to purple when tested on an oxidase paper (Figure 1E). In the indole test, all isolates showed the presence of a red ring after being dripped with Kovacs reagent (Figure 1F).

Molecular testing through PCR revealed that 9 out of 13 suspected isolates were confirmed as *P. multocida* serotype A with an amplification size of 1044 bp using specific capA primers (Figure 2).

Antibiotic sensitivity by disk diffusion method

A total of 9 out of 13 isolates, which were confirmed positive for *P. multocida* serotype A through PCR testing, were then subjected to sensitivity testing using the disk diffusion method. The sensitivity test results were evaluated based on the formation of inhibition zones (Figure 3). Isolates tested via the disk diffusion method exhibited different resistance patterns. Each isolate's resistance profile was compared against Clinical and Laboratory Standards Institute (CLSI) standards (2015) for amoxicillin, tetracycline, and sulfamethoxazole, CLSI standards (2015) for enrofloxacin, and European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards 2024) for ciprofloxacin and colistin.

The sensitivity patterns exhibited by each isolate varied, as shown in Table 3. The isolate with the freeze-dried ampoule code B001 demonstrated the highest level of resistance, while the isolates with ampoule codes B0018, B020, B073, and B077 showed the lowest resistance. The antibiotics used in the disk diffusion test exhibited different sensitivities. Table 4 shows that three isolates were resistant to ciprofloxacin, three were resistant to amoxicillin, and one isolate was resistant to tetracycline, enrofloxacin, and lincomycin. The antibiotic resistance patterns indicate the presence of multidrug resistance in several tested isolates. Specifically, two isolates exhibited resistance to more than three types of antibiotics, as illustrated in Table 5.

Table 2. Culture results, identification, and confirmation of *Pasteurella multocida* from Hisex Brown layer chickens using various test methods

No.	Isolate code	Macroscopic	Gram staining	MCA	Catalase Test	Oxidase Test	Indole Test	PCR CapA
1	B001	+	+	–	+	+	–	+
2	B008A	+	+	–	+	+	+	+
3	B009A	+	+	–	+	+	+	–
4	B010A	+	+	–	+	+	+	–
5	B018	+	+	–	+	+	+	+
6	B020	+	+	–	+	+	+	+
7	B036	+	+	–	+	+	+	+
8	B071	+	+	–	+	+	+	+
9	B072	+	+	–	+	+	+	–
10	B073	+	+	–	+	+	+	+
11	B074	–	–	+	–	–	+	–
12	B075	+	+	–	+	+	+	+
13	B076	+	+	–	+	+	+	–
14	B077	+	+	–	+	+	+	+

Isolate code: Bacterial isolate that coded in freeze-dried ampoules; MCA: Mac Conkey Agar

Table 3. Sensitivity pattern of *Pasteurella multocida* from Hisex Brown layer chickens to antibiotics

Sample code	Susceptibility of *Pasteurella multocida* to antibiotics						
	TE	AML	ENR	SXT	LCS	CIP	CT
B001	R	R	R	S	S	R	R
B008A	S	S	S	S	S	R	R
B018	S	S	S	S	S	S	R
B020	S	S	S	S	S	S	R
B036	S	S	S	S	S	R	R
B071	S	R	S	S	S	S	R
B073	S	S	S	S	S	S	R
B075	S	R	S	S	R	S	R
B077	S	S	S	S	S	S	R

TE: Tetracyclin; AML: Amoxicillin; ENR: Enrofloxacin; SXT: Sulfamethoxazole; LCS: lincomycin; CIP: Ciprofloxacin; CT: Colistin; S: sensitive; R: resistance

Table 4. Number of *Pasteurella multocida* isolates from Hisex Brown layer chickens in West Java, Indonesia that are resistant to antibiotics

Antibiotics	Number of isolates based on resistance category		
	Sensitive	*Intermediate*	Resistance
Tetracyclin	8	0	1
Amoxicillin	6	0	3
Enrofloxacin	8	0	1
Sulfamethoxazole	9	0	0
Lincomycin	8	0	1
Ciprofloxacin	6	0	3
Colistin	0	0	9

Table 5. Antibiotic resistance based on the number of *Pasteurella multocida* isolates that cause fowl cholera Hisex Brown layer chickens

Amount of Antibiotic	Amount of isolates[a]	Antibiotics [b]
1	4	CT
2	2	CIP, CT
	1	AML, CT
3	1	AML, LCS, CT
4	0	-
5	1	TE, AML, ENR, CIP, CT
6	0	-
7	0	-

[a] isolates that are resistant to antibiotics. [b] resistance to ≥ 3 types of antibiotics is referred to as multiresistance. TE: Tetracyclin; AML: Amoxicillin; ENR: Enrofloxacin; SXT: Sulfamethoxazole; LCS: lincomycin; CIP: Ciprofloxacin; CT: Colistin

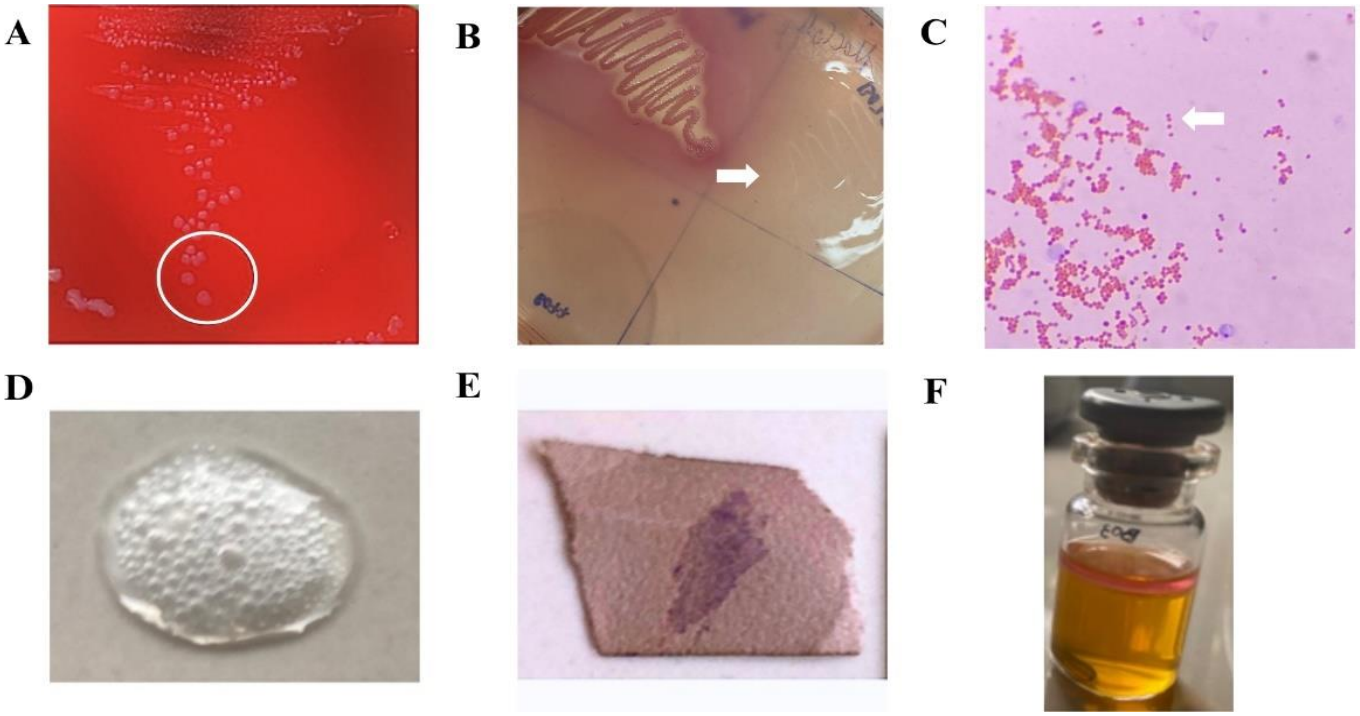

Figure 1. Culture and identification results using various methods. **A:** Morphology of *Pasteurella multocida* colonies on blood agar media (circle); **B:** Absence of bacterial colonies on MacConkey Agar media (arrow); **C:** *Pasteurella multocida* bacteria observed under the microscope (magnification 400x) (arrow); **D:** Catalase test showing bubble formation when *P. multocida* reacts with H_2O_2; **E:** Purple color formation due to *P. multocida* streaks on the oxidase paper; **F:** Red ring in the indole test confirming *P. multocida* isolate

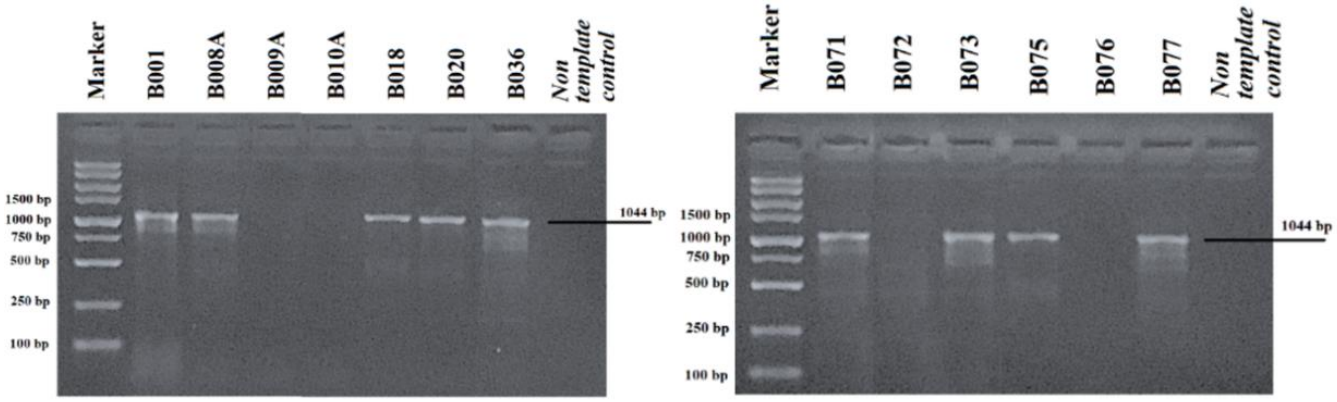

Figure 2. PCR results targeting 1044 bp against *Pasteurella multocida* isolates. Marker: VC 100 bp Plus DNA Ladder Vivantis

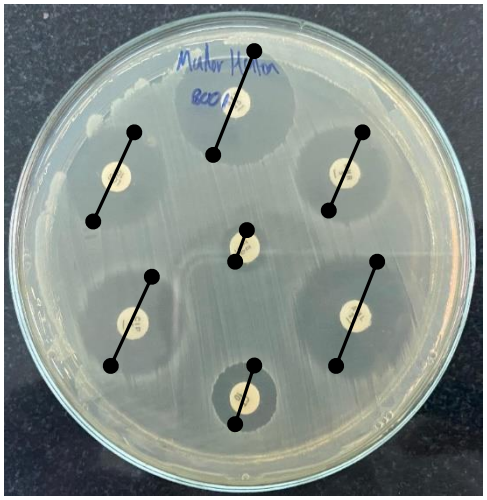

Figure 3. Measurement of the diameter of the inhibition zone in the disk diffusion test. Line marks with rounded edges indicate the apparent diameter of the inhibition zone

DISCUSSION

Fowl cholera has been identified as a significant concern in the commercial poultry business, prompting the use of various techniques to investigate the diversity and transmission patterns of *P. multocida* strains globally (Subaaharan et al., 2010).

Serotypes A, D, and F of *P. multocida* have enzymes capable of producing glucuronic acid and glucosamine, which are modifications of hyaluronic acid, whereas *P. multocida* type B lacks the *hyaC* and *hyaD* genes (Pasomboon et al., 2021). These genes are critical in the synthesis of glucuronic acid and hyaluronic acid. According to Guan et al. (2020), the difference between *P. multocida* serotypes A, D, and F, and type B lies in their capsular components. Serotypes A, D, and F consist of glycosaminoglycan (GAG), while type B consists of non-GAG-like components.

Antibiotic resistance in bacterial infections is a significant global challenge (Frieri et al., 2017). Based on its mechanisms, antibiotic resistance is classified into four categories, including modification of antibiotic molecules, preventing antibiotics from reaching their targets, bypassing antibiotic targets, and cell adaptation to antibiotics (Sabtu et al., 2015; Munita and Arias, 2016).

The antimicrobial resistance patterns in this study align with those of some previous research. Sarangi and Panda (2011) studied the antibiotic sensitivity test of *P. multocida* isolates and found that the organisms were sensitive to enrofloxacin. In the current study, eight out of nine isolates showed sensitivity to enrofloxacin, a fluoroquinolone antibiotics commonly used as a broad-spectrum antibiotic class for various infections (Redgrave et al., 2014). A study by Furian et al. (2014) indicated high antibiotic resistance to enrofloxacin. Resistance to the quinolone group can occur due to type IV topoisomerase

mutations targeting these antibiotics (Redgrave et al., 2014). Another quinolone that was tested in the present study was ciprofloxacin. In this study, six out of nine isolates were sensitive to ciprofloxacin as another quinolone.

In contrast to Sarangi and Panda (2011), the organisms in the current study were sensitive to sulfamethoxazole, an antibiotic widely used in humans and commonly used to treat bacterial infections in pigs and cattle (Vila-Costa et al., 2017). Resistance can occur through several mechanisms, including changes in membrane permeability, less sensitive enzymes, changes in bacterial enzyme targets, mutations in enzyme targets, and inherent resistance (Huovinen, 2001).

Shivachandra et al. (2004) reported significantly elevated levels of resistance (tetracycline 24.39%) in a study that examined 123 strains of *P. multocida*. These strains were collected from outbreaks of fowl cholera in different avian hosts in various regions of India. In this study, eight out of nine isolates were sensitive to tetracycline.

In the present study, six out of nine isolates were sensitive to amoxicillin. A study by Dieb et al. (2020) indicated high resistance of *P. multocida* isolates to amoxicillin. Resistance to beta-lactam antibiotics occurs when PBP undergoes modification or structural changes. Penicillin-binding protein (PBP) is an enzyme that plays a crucial role in the biosynthesis of bacterial cell walls as a peptidoglycan precursor (Halawa et al., 2023).

Lincomycin and colistin were also among the antibiotics examined in the present study. In Table 4, eight out of nine isolates showed sensitivity to lincomycin. Lincomycin is a lincosamide antibiotic derived from several *Streptomyces* (*S.*) species, such as *S. lincolnensis*, *S. roseolu*, *S. caelestis*, and *Micromonospora halphytica*. Lincosamide antibiotics are commonly used as therapeutic agents against anaerobic bacterial infections and some protozoan species. These antibiotics work by inhibiting bacterial protein synthesis, slowing bacterial growth, or killing the bacteria (Spízek et al., 2004). The antibiotic activity against *Pasteurella multocida* indicates fairly good sensitivity. Resistance mechanisms can occur in three ways including modification of antibiotic targets, bacterial efflux pumps, and drug inactivation (Leclercq, 2002).

All isolates examined in the current study displayed resistance to colistin. This finding aligns with a study by El-Demerdash et al. (2023), which reported that 60% of *P. multocida* isolates from birds were resistant to colistin. The primary mechanism of resistance to colistin is typically a chromosomal mutation in genes related to altering the lipid A of lipopolysaccharides (LPS). Such modifications alter the target site of colistin, serving as an adaptive defense mechanism against the antibiotic.

The results of resistance tests indicated that several isolates exhibit multidrug resistance patterns, as shown in Table 5. Multidrug resistance patterns complicate the treatment of bacterial infections using antibiotics (Frieri et al., 2017). Bacterial multidrug resistance to antibiotics can arise from the accumulation of plasmid or transposon genes that confer resistance (R-plasmids) to a particular antibiotic and/or from efflux pumps expelling more than one type of antibiotic (Nikaido, 2009). In addition, the presence of small plasmids has been associated with antimicrobial resistance in *P. multocida* (Rosenau et al., 1991). The simultaneous presence and dissemination of these small plasmids have led to the development of *P. multocida* isolates with multi-resistance (San Millan et al., 2009) and specific resistance to ampicillin (Rosenau et al., 1991), tetracycline (Kehrenberg et al., 2001), and streptomycin (Wu et al., 2003).

CONCLUSION

The isolation and identification of suspected fowl cholera cases in Hisex Brown layer chickens from farms in Sukabumi, West Java, indicated that 13 out of 14 isolates are positive for *Pasteurella multocida*, with 9 out of 13 isolates positive for *P. multocida* serotype A. Antibiotic resistance testing revealed that all nine isolates of *P. multocida* serotype A were resistant to colistin. Still, some isolates remained sensitive to tetracycline, amoxicillin, enrofloxacin, sulfamethoxazole, lincomycin, and ciprofloxacin, with two isolates showing multidrug resistance patterns.

DECLARATIONS

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Authors' contributions

Agustin Indrawati acquired the funds, conceptualized and supervised the work, and revised the manuscript. Titiek Sunartatie, Safika, Herjuno Rafi Abhirama, Citra, Ryan Septa Kurnia, Muhammad Ade Putra, Christian Marco Hadi Nugroho, and Ni Luh Putu Ika Mayasari conducted the experiments, collected and analyzed the data, and prepared the manuscript. All authors read and approved the last manuscript version.

Competing interests

The authors declared that there are no competing interests.

Ethical considerations

The authors declare that this manuscript is original and is not being considered elsewhere for publication. Other ethical issues, including consent to publish, misconduct, fabrication of data, and redundancy, have been checked by the authors.

Availability of data and materials

All current study's data are available upon reasonable requests from the authors.

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