Pathogenicity of *Clostridium perfringens* Philippine Isolate in Necrotic Enteritis Across Broiler Growth Stages

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Abstract

Clostridium perfringens was isolated from Philippines broiler chickens of a local farm exhibiting clinical signs of necrotic enteritis. This local isolate induced necrotic enteritis (NE) experimentally in susceptible broiler chickens to demonstrate the disease and the lesions it would produce. Experimental chickens were subjected to stress such as vaccination and pathogenic *Escherichia coli* and *Salmonella enteritidis*. Seven treatment groups involving various combinations of the above agents were used to demonstrate lesions of NE. Gross intestinal lesion scoring was performed at necropsy on the 3rd, 7th, 11th, 14th, and 18th-day post-infection, depending on the treatment group. Results showed that the local C. perfringens isolate was able to cause lesions of enteritis but did not demonstrate the classic towel-like lesion of NE, as described by the literature. The treatment combination of *C. perfringens* and *S. enteritidis* produced the highest intestinal lesions scores. However, *C. perfringens* alone can experimentally induce enteritis with a lesser severity. This is the first report of experimental induction of NE in broiler chickens using a local *C. perfringens* isolate in the Philippines.

Keywords: antibiotic-free, infectious disease, necrotic enteritis, pathogenic bacteria, post-infection

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INTRODUCTION

Livestock production in general and domestic chicken production in particular plays a vital socio-economic role for people living in low-income countries of Africa and Asia (Moazeni *et al*., 2016[a]). Domestic chickens are widely distributed avian species around the world, due to their short generation interval and adaptability in a wide range of agro ecologies (Mohammadifar and Mohammadabadi, 2018; Moazeni *et al*., 2016[b]; Khabiri *et al*., 2022). The domestic chickens provide high quality protein and income for the poor rural households and are the most widely kept livestock species in the world (Mohammadifar and Mohammadabadi, 2018). This is due to the presence of the valuable traits of chicken like disease resistance, adaptation to harsh environments and ability to utilize poor quality feeds (Shahdadnejad *et al*., 2016; Khabiri *et al*., 2023).

The broiler industry in the Philippines continues to expand from 2009 to 2018, the average annual growth rate was 40% and had developed into a large-scale industry during the past two decades (Natalie Berkhout, 2020). The development was determined by multiple factors, including infectious diseases caused by bacteria, viruses, fungi, and parasites that could affect the growth of broiler chickens resulting in profit loss for the farmers (Hafez and Attia, 2020). Over the last 50 years, sub-therapeutic doses of antibiotics in feed have become a reliable tool for increasing the productivity of chickens by controlling bacterial pathogens. However, increasing concern about the occurrence of antibiotic-resistant bacteria in poultry meat has halted the use of antibiotics in poultry feeds globally due to the emergence of antibiotic resistance in microbial communities poses a global threat to food safety and security (Okaiyeto *et al*., 2024).

Necrotic Enteritis (NE), caused by *Clostridium perfringens*, is an important infectious disease the broiler industry is suffering from globally (Hofacre *et al*., 2018). NE is a major challenge in antibiotic-free chicken production. Broiler chickens with NE manifest diarrhea, and decrease feed intake resulting in decreased weight gain, feed conversion, increased mortality rates (M'Sadeq *et al*., 2015) and increased zoonotic risk of contamination of poultry products for human consumption (Chang *et al*., 2024). Incidences are increasing because of the global regulatory bans on antimicrobials and the decline in the use of ionophores coccidiostats resulting in losses due to high mortality.

Necrotic enteritis is difficult to reproduce experimentally with *C. perfringens* alone. Various factors have been identified to promote the development of diseases, including stress, nutrition, coccidiosis, and other diseases that may disturb the physiology of the intestines (Fathima *et al*., 2022). In most experimentally-induced NE, coccidia was given to broiler chickens prior to *C. perfringens* infection, because it is the most commonly known predisposing factor of the disease (Feng *et al*., 2022). However, any of the predisposing factors of NE mentioned above may still be used to experimentally-induce the disease.

The presence of *C. perfringens* in Philippine broiler chickens will affect current growth trends of this industry. With the current intensive husbandry practices and observation of antimicrobial withdrawal period prior to market, it is significant to demonstrate the presence of the organism and its effects with regards to lesions and other stress factors. The use of a local isolate in experimental chickens can be used to guide veterinarians and farm workers in familiarizing themselves in disease diagnosis of *C. perfringens* in broiler chickens especially in Philippines. The study was conducted to isolate *C. perfringens* from the intestines of broiler chickens with signs of enteritis and demonstrate the disease it causes in susceptible broiler chickens.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños.

Study Period and Location

The study was conducted at the Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, College, Laguna, from December 2022 to March 2023.

Primary Isolation of *C. perfringens*

Broiler chickens at 32–37 days old (market age) were used for the isolation of *C. perfringens*. These chickens were from flocks having signs of enteritis demonstrated as weight loss, watery diarrhea, pasted vents, and high mortality. Intestinal samples were obtained at necropsy. These were swabbed and minced aseptically then swabs were inoculated in blood agar plates (Acumedia®, USA) while the minced intestines were inoculated to cooked meat broth (HiMedia®, India). Colonies with hemolysis in blood agar plates (BAP) were Gram-stained and purified to another blood agar plate. Colonies with double-zone hemolysis were picked and transferred in a cooked meat broth for propagation and storage.

Confirmation from pure cultures was performed by Thioglycollate broth (HiMedia®, India), biochemical tests and reverse CAMP (Christie-Atkins-Munch-Peterson) test. The biochemical tests that were used for confirmation were gelatinase test (Pronadisa®, Madrid), carbohydrate fermentation tests (glucose, lactose, sucrose, and maltose) with phenol red base (Pronadisa®, Madrid), and litmus milk (BBL™, Canada).

Reverse CAMP test was also used as a final confirmatory test for the identification of *C. perfringens. C. perfringens* was streaked vertically at the center of a BAP and *Streptococcus agalactiae* was streaked perpendicularly close to *C. perfringens* at the

center of the plate without the two organisms touching each other. Individually, both organisms produce partial hemolysis in BAP, but in the reverse CAMP test, complete hemolysis would be observed near the intersection of the two organisms. Usually, the appearance of the complete hemolysis has a bow-tie appearance. When this occurs, the isolate is further confirmed as *C. perfringens.*

Inoculation of *C. perfringens* to Susceptible Broiler Chickens

Ninety-six-day-old chicks day-old Cobb X Cobb male broiler chicks were used for experimental infection and then randomly assigned to treatment groups as described in Table 1.

Salmonella enteritidis and *Escherichia coli* were used as predisposing factors to experimentally induce NE. *S. enteritidis* and *E. coli* were local field isolates and were used in combination with *C. perfringens* in several treatments. These stressors were given to immunocompromise the susceptible chickens and were used as co-infection agents to experimentally induce NE.

S. enteritidis and *E. coli* were propagated in nutrient broth (EcoBio®, biolab, Hungary), for *S. enteritidis* and *E. coli*, and cooked meat broth was used for the propagation of *C. perfringens*. The concentration of each bacteria was adjusted prior to each inoculation schedule. *S. enteritidis* and *E. coli* in nutrient broth were adjusted to approximately 1×10^6 CFU/mL (Gao *et al*., 2021) while *C. perfringens* in cooked meat broth was adjusted to approximately 1×10^9 CFU/mL by serial dilution using a McFarland nephelometer standard (Jesudhasan *et al*., 2021) with modification.

Each inoculum was given to the chickens at approximately 1 mL of each organism by oral gavage. Vaccine application was administered to T1 and T2 at 17 days of age. The vaccine combination of Newcastle Disease (ND) and Infectious Bronchitis (IB Mass) by Hipra Philippines was used as a stressor to further immunocompromise the birds. The inoculation is carried out at ages 17, 21, 22, and 23 due to the schedule of vaccine application and foccused on the growth.

After oral induction of *C. perfringens*, the broiler chickens were euthanized for the observation of intestinal gross lesions. Necropsy schedule were at Day 20, 28 and 35, depending on the time of infection per treatment (Table 1). Gross intestinal lesions were graded (0–4) according to its severity. These lesions are described in Figure 1, wherein grade 0 had no lesions while grade 4 is the most severe with massive hemorrhage on the entire length of the gastrointestinal tract. Figure 1 also shows examples of how the intestinal lesions were graded.

Data Analysis

The results of this study are presented descriptively in the form of figures and tables.

Table 1. Description of treatment groups and the schedules for inoculation and necropsy

Group (n per group = 12)	Description	Inoculation schedule (based on age)	Necropsy schedule (based on age)		
			Day 20	Day 28	Day 35
T1	CP + vaccine	Day 17	Yes	Yes	Yes
T2	EC + vaccine	Day 17	Yes	Yes	Yes
T3	CP + EC	Day 17	Yes	Yes	Yes
T4	SE only	Day 21	ND	Yes	Yes
T5	CP + EC	Day 21, 22, 23	ND	Yes	Yes
T6	CP + SE	Day 21, 22, 23	ND	Yes	Yes
T7	CP only	Day 21, 22, 23	ND	Yes	Yes
Control	No inoculum	None	ND	Yes	Yes

CP = *C. perfringens*; ND = No necropsy done; SE = *S. enteritidis*; EC = *E. coli*.

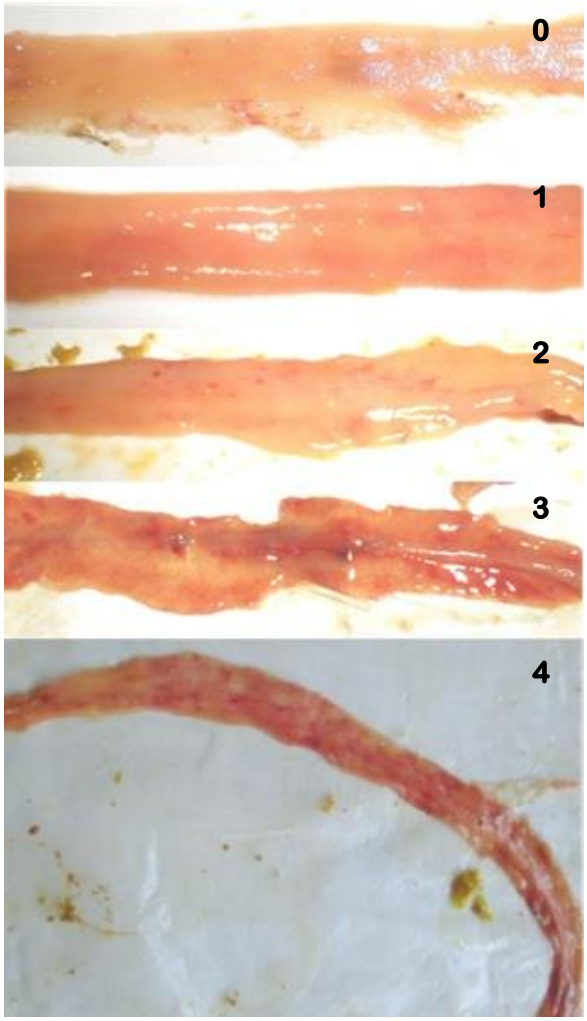

Figure 1. Gross intestinal lesion grading according to Alnassan *et al*., (2014) and Raman *et al*., (2011) for necrotic enteretidis (NE)-typical lesions. 0 = no gross lesion; 1 = thin-walled or friable small intestine; 2 = focal necrosis or ulceration; 3 = larger patches of necrosis; 4 = severe, extensive necrosis typical of field cases.

RESULTS AND DISCUSSION

Primary Isolation of *C. perfringens*

C. perfringens was isolated from a local broiler chicken. The isolate exhibited distinct characteristics of *C. perfringens* such as the double-zone hemolysis on BAP, positive for catalase, gelatinase, and sugar fermentation (glucose, sucrose, lactose and maltose), anaerobic growth in Thioglycollate broth, stormy fermentation in litmus milk and finally and a reverse CAMP reaction (Figure 2). The classic Gram-positive drumstick-like appearance with endospores bulging subterminally from the vegetative cell was also the characteristic *C. perfringens* microscopic morphology observed.

Isolation of *C. perfringens* in the Philippines was initially reported by Maluping (Maluping *et al*., 2003). Forty *C. perfringens* samples were isolated from one hundred fifty broilers from randomly selected farms. A similar study by Olkowski *et al*. (2006) in Canada reported in 2005 the isolation of *C. perfringens* in field cases. Study by Craven *et al*. (2001) in US detected the *C. perfringens* in fecal or cecal samples, 94% of the flocks became positive for this bacterial enteropathogen, and only one remained negative throughout the 6-to-8-wk rearing period *C. perfringens* was again isolated from Philippine broilers in this study, despite the limited farm sample (3 farms), Thus, *C. perfringens* was still present in the local farm with a history of clinical signs of NE.

The current isolation methods used were compared to the previous local study. Several isolation techniques were similar except that instead of only obtaining intestinal swabs, aseptic mincing of the intestines was performed and eventually placed in cooked meat broth (Habib Wani *et al*., 2017). From cooked meat broth, the organism was later transferred to BAP. The prior step improved conditions for the selective growth of *C. perfringens* which would allow it to propagate faster than other gut organisms. Other similarities performed are the confirmation of isolates which included the use of biochemical tests and Thioglycollate broth, to confirm the anaerobic nature of the organism (Hanif *et al*., 2015). In addition to this, Maluping *et al*., (2003) used litmus milk to confirm the identity of the isolates. To make a more definitive identification of *C. perfringens*, a reverse CAMP test was included to confirm the identity of the current *C. perfringens* isolate.

Necrotic enteritis has been reported to be preceded by mild intestinal coccidiosis (Adhikari *et al*., 2020). This was not included in our treatment protocols because of the non-availability of *Eimeria sp.* challenge organism at the time of the study. However, Timbermont *et al.*

2011 stated that NE may also occur with other predisposing factors such as those included in this experimental design. However, a study by Mohiuddin *et al*. 2021 report that virulent *C. perfringens* type G strains can induce NE lesions in the absence of other predisposing factors. Birds in the clostridia-challenged group showed moderate to severe NE lesions.

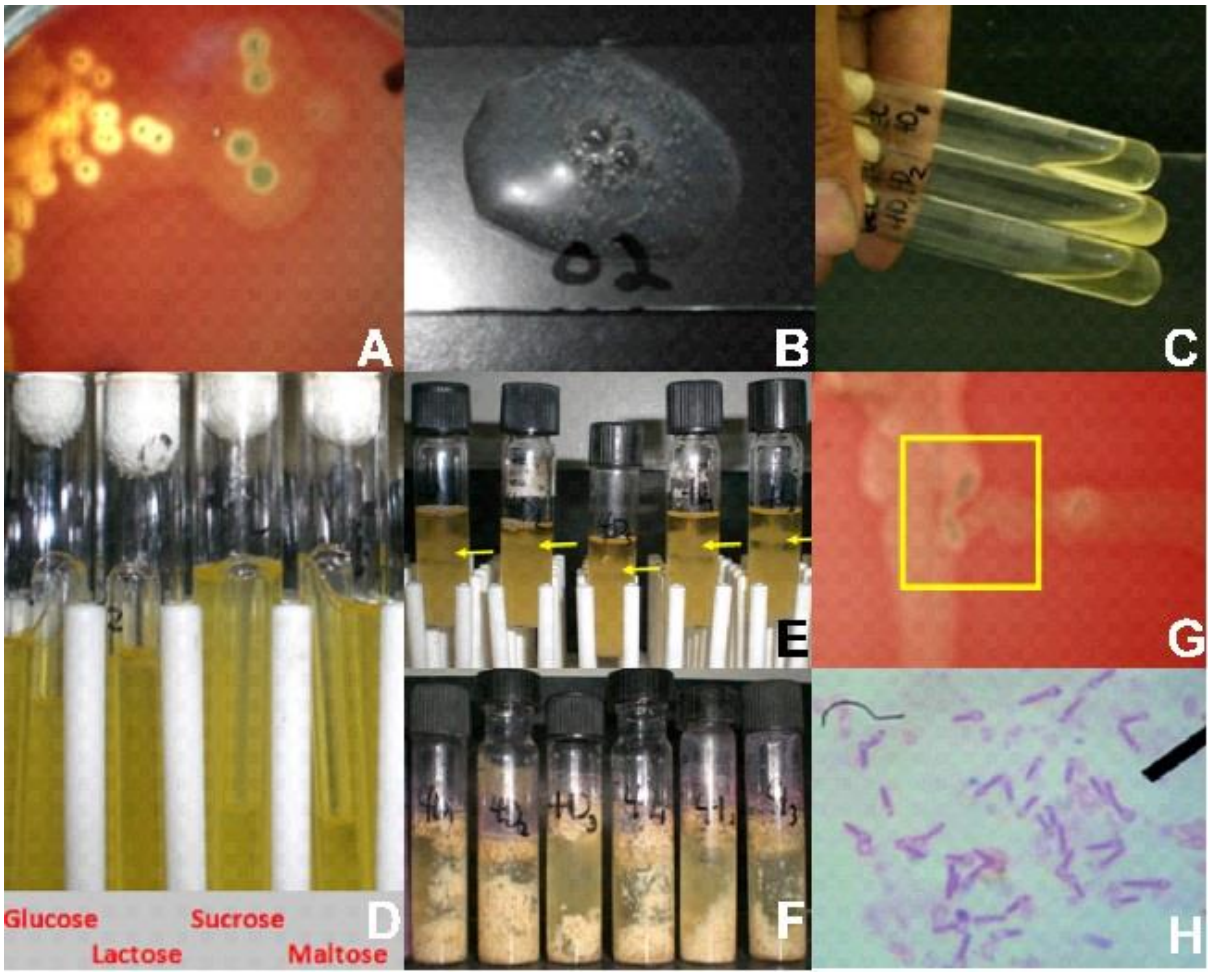

Figure 2. A – Double-zone hemolysis on BAP; B – Catalase test positive; C – Gelatinase test positive; D – Sugar fermentation test positive (glucose, sucrose, lactose, maltose); E – Anaerobic growth in thioglycollate broth; F – Stormy fermentation in litmus milk; G – Reverse CAMP test reaction; H – Gram-positive large rods with subterminal endospores.

Table 2. Ranking of treatment groups based on severity and lesion scoring

	Rank 1	**Rank 2**	**Rank 3**	**Rank 4**	**Rank 5**	**Rank 6**	**Rank 7**
Day 20	T3 (1.25)	T2 (1.0)	T1 (0.50)	N/A	N/A	N/A	N/A
Day 28	T6 3× gavage (2.2)	T7 3× gavage (2.0)	T5 3× gavage (1.83)	T2 and T3 (1.5)	T4 (1.4)	Control (1.33)	T1 (1.25)
Day 35	T6 3× gavage (3.0)	T4 (2.6)	T2, T3 and T5 3× gavage (2.0)	T7 3× gavage (1.66)	T1 (1.3)	Control (1.0)	

CP = *C. perfringens*; SE = *S. enteritidis*; EC = *E. coli*; N/A = not available.

Table 3. Gross intestinal lesion scoring of experimentally infected birds per treatment group

Average Lesion Score			
Group	**Day 20**	**Day 28**	**Day 35**
T1	0.5	1.25	1.3
T2	1	1.5	2
T3	1.25	1.5	2
T4	N/A	1.4	2.6
T5	N/A	1.83	2
T6	N/A	2.2	3
T7	N/A	2	1.66
Control	N/A	1.33	1

CP = *C. perfringens*; SE = *S. enteritidis*; EC = *E. coli;* N/A = not available.

Table 4. Summary of re-isolated bacteria from the intestines of experimentally infected chickens

	Day 20			**Day 28**			**Day 35**		
Group	**CP**	**EC**	***Salmonella* sp.**	**CP**	**EC**	***Salmonella* sp.**	**CP**	**EC**	***Salmonella* sp.**
T1	1/4	N/A	N/A	2/2	2/2	0/2	0/2	2/2	0/2
T2	0/4	N/A	N/A	0/2	2/2	0/2	0/2	2/2	0/2
T3	1/4	N/A	N/A	0/2	2/2	0/2	0/2	2/2	0/2
T4			ND[1]	0/2	2/2	0/2	0/2	2/2	1/2
T5			ND[1]	0/2	2/2	0/2	0/2	2/2	0/2
T6			ND[1]	0/2	1/2	1/2	0/2	2/2	1/2
T7			ND[1]	0/2	2/2	0/2	0/2	2/2	1/2
Control			ND[1]	0/2	2/2	0/2	0/2	1/2	1/2

CP = *C. perfringens*; SE = *S. enteritidis*; EC = *E. coli;* N/A = not available; ND = No necropsy done.

Inoculation of *C. perfringens* to Susceptible Broiler Chickens

Mortalities that occurred prior to necropsy dates were not included in the gross intestinal lesion scoring. All the remaining birds were necropsied in their scheduled date and intestines were scored. On Day 20 (first necropsy), samples from T1, T2 and T3 were obtained. Gross intestinal lesions scoring (Table 2) showed samples from T3 developed the most severe enteritis, three days post-infection. This was followed by T2 and lastly, T1. On the second necropsy schedule, Day 28, the enteritis was ranked from most severe to mildest as follows: T6, T7, T5, T2 and T3, T4, Control, and T1.

On the third necropsy schedule, Day 35, the severity of enteritis was again ranked from most severe to mildest and are as follows: T6, T4, T2, T3 and T5, T7, T1 and Control. For the last two necropsy dates, T6 comparatively had the most severe lesions.

It was also observed that the gross intestinal lesion scores of all treatment groups except for T7 was increasing from the first necropsy date (Day 20) to the last necropsy date (Day 35) (Table 3). This pattern suggests disease progression caused by *C. perfringens* and other stressor bacteria over time.

Mortalities that were observed during the experiment were attributed to the handling during oral gavage, heat stroke and/or anaphylaxis or toxemia due to the inoculum given. *C. perfringens* was only re-isolated from four birds in T1 on Day 20 and 28, and T3 on Day 20. Re-isolation was seen in the early necropsy dates only. Similarly, *S. enteritidis* was also isolated from a few birds and majority of the re-isolated *S. enteritidis* was in the later dates of necropsy, including one from the control group. *E. coli* was isolated from all samples processed for the isolation of *E. coli* except in two birds from T6 and the control group (Table 4).

Experimental induction of NE using a local isolate was performed by additional stressors such as vaccination and other intestinal bacterial pathogens. These were given to reduce immunity and increase the susceptibility of experimental birds. All seven treatment conditions elicited lesions in the experimental birds. Lesions ranged from thinning of the intestinal wall to diffused hemorrhages of the mucosa. Lesions induced by Olkowski *et al*. 2006 using Canadian isolates and feed di*et al*terations included hemorrhages that were slightly similar to the lesions produced in all seven treatment groups earlier described. In addition, these workers observed focal to multi-focal, extensive or disseminated hyperemia and hemorrhages. However, additional observations of yellow or greenish, loosely adherent material lining the intestinal mucosa were reported. They described these lesions to resemble diphtheritic pseudo-membrane, such lesions were not observed in the experimentally infected local birds. On the other hand, Keyburn *et al*. 2010 in Australia observed the distinct towel-like lesion, the most characteristic lesion of NE. This was not observed in the intestines examined.

The production of lesions is due to the *C. perfringens* toxins produced by the organism. According to Goossens *et al*., (2017), the alpha toxin which is produced by all *C. perfringens* strains is essential for necro-haemorrhagic enteritis and the major toxin involved in NE because it is responsible for attacking the cell lining of the intestines resulting in cell and tissue damage. However, Lee and Lillehoj, 2021 reported that strains of *C. perfringens* without the alpha-toxin were still virulent leading them to the discovery of another toxin that was a key virulence determinant in *C. perfringens*: the Necrotic Enteritis B-like (NetB) toxin. The occurrence of $NetB^+$ *C. perfringens* strains has been increasingly reported in NE-afflicted poultry flocks globally.

The major toxins produced by *C. perfringens* are alpha (CPA), beta (CPB), epsilon (ETX), iota (ITX), enterotoxin (CPE), and necrotic B-like (NetB) toxins. In most cases, host–toxin interaction starts on the plasma membrane of target cells via specific receptors, resulting in the activation of intracellular pathways with a variety of effects, commonly including cell death. In general, the molecular mechanisms of cell death associated with C. perfringens toxins involve features of apoptosis, necrosis and/or necroptosis (Navarro *et al*., 2018).

The lesions observed differed from those described by Olkowski *et al*. 2006 and Keyburn *et al*. 2010 mainly due to the strain used to experimentally induce the disease. The locally isolated strain perhaps lacks the NetB toxin to produce the mentioned diphtheritic pseudo-membrane, but it was still able to produce enteritis in susceptible broiler chickens (Navarro *et al*., 2018; Kartikasari *et al*., 2019). Mortalities in all the treatment groups with *C. perfringens* were noted, with the highest mortalities seen in the *C. perfringens* + *E. coli* treatment groups; the most damaging lesions based on lesion scoring were in the *C. perfringens* + *S. enteritidis* treatment group. Determination of the type of toxin produced by the local *C. perfringens* isolates would be key to explaining the difference in the tissue damage observed.

Enteric lesions were most severe in treatments where *C. perfringens* was in combination with *S. enteritidis*. *S. enteritidis* alone can cause severe lesions; however, severity was amplified with the presence of *C. perfringens*. Despite enteric lesions in the treatment groups where *C. perfringens* was inoculated, the bacterium was re-isolated from only a few birds. Majority of the re-isolated *C. perfringens* were from the early necropsy dates specifically from T1 wherein vaccination and *C. perfringens* only was given. *C. perfringens* was re-isolated 3- and 11-days post-infection. No *C. perfringens* was isolated from all treatment groups in the third necropsy (10- and 18-days post-infection) which could be accounted to the overgrowth of Enterobacteriaceae.

The *E. coli* isolated in treatment groups cannot be distinguished from normal *E. coli* inhabitants of the gut. This organism is a fast grower and less fastidious than *C. perfringens* and *S. enteritidis*, given the environment it needs for growth. *E. coli* is impossible to ignore in the livestock industry (Peek *et al*., 2018).

Contamination of control groups indicates cross contamination which was attributed to several factors such as the set-up of the experimental houses and cages, the handling of the chickens and equipment during inoculation of the bacteria, and/or handling of fecal material, housing, and management of the flocks. The set-up used could be further improved by using isolated cages and rooms for experimental induction of infectious and/or contagious diseases, and by assigning individual rooms with exactly the same environmental conditions for each treatment group. Despite the limitations, the isolated *C. perfringens* was found pathogenic and capable of producing enteritis with hemorrhages. However, more severe lesions were observed when pathogenic *E. coli* and *S. enteritidis* were included.

CONCLUSION

C. perfringens was isolated from a broiler farm with clinical signs of watery diarrhea, pasted vents and high mortality. The isolation in this study was confirmed based on the published morphological and biochemical reactions of *C. perfringens*. Enteritis was observed when the local *C. perfringens* isolate was inoculated to susceptible chicks, however, the lesions observed were not the characteristic towel-like lesion of NE. Enteritis was observed in all treatments involving *C. perfringens* but the combination of *C. perfringens* and *S. enteritidis* (T6) produced the most severe lesions based on intestinal lesion scores. Mortalities were highest in the treatment with a combination of *C. perfringens* and *E. coli* (T3). The local *C. perfringens* isolate should be further characterized and the toxin it produces should be determined. It is also recommended that the depth of intestinal layer damage be evaluated through histopathological examination of the intestines. Other diagnostic methods could be developed for further disease diagnosis. This is the first report of experimental induction of NE in broiler chickens using a local *C. perfringens* isolate in the Philippines.

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AUTHORS' CONTRIBUTIONS

MRSR: Conceptualization, Formal Analysis, Resources, Validation, Investigation, Writing – original draft. LP: Visualization, Writing –review and editing. JPO, HGR: Data curation, Formal Analysis. JFC: Conceptualization, Supervision, Data curation and Validation. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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