

***Helicobacter pullorum* in broiler chickens and the farm environment: A One Health approach**

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Abstract

Aim: This study aimed to investigate the occurrence of *Helicobacter pullorum* in broiler chickens and their farm environment.

Materials and Methods: The ceca from 100 broiler chickens from ten farms were sampled from processing sites or markets. The cecal contents were aseptically collected from each cecum and cultured. The farms were visited, and environmental samples were collected which included water, house flies, floor swabs and soils in chicken houses.

Results: *H. pullorum* was present in 51% of the broilers; 17.5% of the flies were found to carry *H. pullorum* and *Campylobacter* spp., 30% of house floors were positive, while all water samples were negative.

Conclusion: Flies could have picked up the organisms from the chickens' feces and/or the environment of the chicken houses or they could be one of the sources in the spread of the organisms. This study also showed that broiler chickens are potential reservoirs for *H. pullorum* and may serve as a source of infection for humans through the food chain.

Keywords: broiler chickens, *Campylobacter*, *Helicobacter pullorum*, house flies.

Introduction

Helicobacter pullorum is a common resident in the ceca of healthy poultry flocks such as broilers, laying hens, turkeys, and birds [1-3]. The organism has also been isolated from laying hens with avian hepatitis [4,5]. The modes by which these broiler chickens become colonized with *H. pullorum* have not been fully understood [6-8]. Several molecular epidemiologic studies showed that *H. pullorum* colonization may occur with a single strain that disseminates throughout the flock [9,10].

Published data are lacking on the survival of *H. pullorum* outside the gastrointestinal niches. *Helicobacter pylori* and *Campylobacter* spp. have been shown to survive in water as viable but non-culturable organisms [11,12], and hence, water may also play a role in the transmission of *H. pullorum* to chickens in the farms. House flies have been reported to carry *H. pylori* and other enteric bacteria such as *Salmonella* [13-15] and *Campylobacter* in poultry flocks [16-18]. The transmission of the organisms is presumed to occur through small quantities of contaminated materials carried on the proboscis, legs, and

body hairs or from materials regurgitated or defecated by the flies. Environmental sources, such as litter, feed, drinkers, and air, have been reported as having a potential role in the transmission of *Campylobacter* in poultry houses [14,19-23]. Ceelen *et al.* [24] isolated *H. pullorum* from farmers' boots. Therefore, environmental sources too may play an important role in the transmission of *H. pullorum* to chickens in the farms. Additionally, *H. pullorum* has also been reported to be associated with gastroenteritis, diarrhea, liver and gall bladder diseases as well as Crohn's disease in human patient [24].

In Malaysia, there is relatively very little information on the occurrence of *H. pullorum* in broiler chickens and the farm environment. Thus, this study aimed to determine the occurrence of *H. pullorum* in broiler chickens in the farm and to detect the presence of *H. pullorum* in the farm environment, namely water, flies, and floors and soils in the chicken houses.

Materials and Methods

Ethical approval

This study was performed as per the guidelines for the care and use of animals by Institutional Animal Care and Use Committee of Universiti Putra Malaysia and Animal Welfare Act.

Sample collection

The intestines of 100 chickens were collected from processing sites or markets. The ceca were carefully and aseptically removed from the intestine, placed in a sterile Petri dish, sealed with Parafilm,

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and kept in a cool box. Visits were made to the farms where the chickens were reared in 10 farms in different locations. Water samples were collected from various sources on the farm, which included tap water, well or groundwater, and drinkers. A total of 40 water samples (100 mL in each bottle) at four samples per farm were collected in sterile bottles. Flies in the poultry farms were collected using Glue Stick Fly Traps. Five flies were pooled in a bottle containing 1 mL of Brucella Broth (BD - Becton Dickinson and Co.). At least 10 flies were caught inside the farm, and another 10 flies were caught outside the farm within 50 m from the chicken houses. Each sample of floor swabs and soils from closed-house farms and floor swab samples from open-house farms were collected in a sterile bottle containing Brucella Broth (BD - Becton Dickinson and Co.). All samples were kept cool during transport to the Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The samples were cultured within 4-6 h after sampling.

Isolation of *H. pullorum*

Fresh cecal contents were obtained aseptically from the ceca of each chicken and subjected to the method of Miller *et al.* [25] which was slightly modified as described in Wai *et al.* [26]. Briefly, approximately 2 g of cecal contents were squeezed into 4 mL (1:2) of 0.85% sterile saline. Then, 100 μ L of the suspension was further diluted in 400 μ L of a sterile enrichment broth mixture (EBM) containing 25 mL of Brucella Broth (BD - Becton Dickinson and Co.), 5 mL of inactivated horse serum (Oxoid, UK), and 7.5 g of glucose (Sigma Chemical Co). Six drops of the aliquot in EMB were placed on 0.45- μ m pore size, sterile cellulose acetate membrane filter (Sartorius) which was earlier placed on 10% sheep blood agar (SBA) (Oxoid, UK) plate and incubated upright in a hydrogen-enriched microaerophilic atmosphere, generated using a gas generating pack BR0038B (Oxoid, UK) without palladium catalyst also recommended by le Roux and Lastovica [27], at 42°C for 1 h to allow passive filtration. Using sterile forceps, the filters were carefully removed, and the plates were incubated at 42°C for 48 h under the same atmospheric conditions as described above. The suspected colonies were selected and subcultured on SBA. *Campylobacter jejuni* ATCC 29428 and *H. pullorum* CCUG 33837 and ATCC 51863 were used as reference strains. All field isolates and reference strains were stored at -20°C and -80°C, respectively.

Isolation of *H. pullorum* from water samples was carried out as per the method described by Diergaardt *et al.* [28]. Briefly, 100 mL of each water sample was filtered; then, the filter was rolled, placed in a sterile bottle containing 9 mL of EBM, and incubated at 42°C for 24 h under similar condition described previously. After incubation, 250 μ L of the culture in EBM was dropped onto the surface of a membrane filter placed

earlier on SBA surface. Similar procedure as described above was used for the isolation of *H. pullorum* from water samples. The pooled samples of flies in each bottle were crushed, and 2 mL of EBM was added. The procedure for the isolation of *H. pullorum* from flies and floor swab samples was similarly carried for cecal contents.

Phenotypic identification of *H. pullorum*

All suspected *H. pullorum* colonies were examined for phenotypic characteristics (Gram-negative, slightly curved, slender rod) and subjected to oxidase, catalase, and indoxyl acetate hydrolysis tests for presumptive identification. The motility of the isolates was determined by phase-contrast microscopy of a wet mount prepared from cultures. Other phenotypic identification was hippurate hydrolysis test, sensitivity to polymyxin B and nalidixic acid, resistance to cephalothin, and growth at 25°C, 37°C and 42°C in aerobic, anaerobic, and microaerobic conditions.

Genotypic identification of *H. pullorum*

A single presumptive *Helicobacter* colony was streaked onto SBA and incubated at 42°C for 48 h under similar atmosphere mentioned above. A few colonies were taken and washed in 1 mL Brucella Broth (BD - Becton Dickinson and Co.), in a 1.5 mL sterile microcentrifuge tube. The extraction of genomic DNA was done per DNeasy Blood and Tissue DNA Purification Kit (QIAGEN). The quality of the extracted DNA was accessed using spectrophotometer and gel electrophoresis. The resulting DNA pellet with desirable quality was stored at -20°C until used. Confirmation of the presumptive isolates was done using a modified species-specific polymerase chain reaction (PCR) assay as described by Miller *et al.* [25]. The primers used were forward primer 5'-ATGAATGCTAGTTGTTGTGAG-3' and reverse primer 5'-GATTGGCTCCACTTCACA-3'. A total of 1 μ L of DNA preparation was added to a 20 μ L (final volume) reaction mixture containing 0.25 μ M of each primer and Maxime PCR Premix Kit (iStar-Taq) (iNtRON Biotechnology, Korea). The PCR product was then incubated with initial denaturation at 94°C for 4 min, followed by 30 cycles of 93°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min with a final incubation at 72°C for 5 min. A 5 μ L of the PCR product was electrophoresed through a 2% agarose gel containing Tris-Borate-EDTA (TBE) buffer (40 mmol/l tris-borate, 2 mmol/l EDTA, pH 7.5) and gel red (3 μ L/mL) in TBE buffer at 75 V, for 80 min. The gel was viewed by ultraviolet (UV) transillumination.

Multiplex PCR (m-PCR) was performed to further identify the presumptive mixed isolates of *H. pullorum* and *Campylobacter* species. The primers used to detect *Campylobacter* were for *Campylobacter* genus specific: Forward C99-GCGTGGAGGATGACACCT and reverse C98-GATTTTACCCCTACACCA and for *H. pullorum* species-specific mentioned as above.

PCR amplifications were performed as described earlier; however, the annealing temperature used was 55°C as mentioned in Shen *et al.* [29].

Results and Discussion

The colonies of suspected *H. pullorum* were very small, pinpoint, translucent, hemolytic, and some were a watery film like. They were Gram-negative, motile (spiral, slow-jerky-tumbling motility under wet mount), and oxidase and mostly catalase positive (isolates from one farm were catalase negative) and all were negative to indoxyl acetate hydrolysis and hippurate hydrolysis tests. Several suspected isolates were observed to show a weak reaction to indoxyl acetate hydrolysis test. All isolates were resistant to cephalothin and sensitive to polymyxin B, and 84.3% were resistant to nalidixic acid. The suspected *H. pullorum* isolates and the reference strain *H. pullorum* ATCC 51863 yielded a single band at 447 bp (Figure-1), whereas the reference strain *C. jejuni* ATCC 24928 did not show any band in the *H. pullorum* species-specific PCR assay. The phenotypic and PCR results provided evidence that the suspected isolates were *H. pullorum*. Of the 100 broiler chickens sampled, 51 (51%) were positive for *H. pullorum*. Pure isolates of *H. pullorum* were obtained from 45 of 51 positive samples from seven farms. One farm (farm 18) had six of ten positive samples which were co-colonized with *Campylobacter* species. Two farms were negative for *H. pullorum*; these two farms (farms 16 and 17) were under closed-housing system (Table-1). In the m-PCR assay, all the suspected isolates showed bands at 447 bp and 296 bp, exactly to that of the reference strain *H. pullorum* ATCC 51863 and *C. jejuni* ATCC 24928, respectively (Figure-2). It was noted that each isolate with weak indoxyl hydrolysis test results showed that it consisted of *H. pullorum* and *Campylobacter* spp. which could not be separated on repeated subcultures.

All the 40 water samples were negative for *H. pullorum* (Table-2). Of the 40 bottles containing pooled fly samples, seven (17.5%) from three farms were positive and found to carry *H. pullorum* and *Campylobacter* spp. (Figure-2 and Table-3). Six (30%) of floor and soil swab samples from three farms were positive and consisted of *H. pullorum* and *Campylobacter* spp. (Figure-2 and Table-3). The flies and floor samples from three farms (9, 11, and 12) were positive for *H. pullorum* and *Campylobacter* spp. Farms 14 and 15 had low occurrences probably due to better environmental conditions.

The study also showed that *H. pullorum* was prevalent in broiler chickens in the farms. Previous studies showed high occurrences of *H. pullorum* in chickens ranged from 60% in the UK [30] to 78.3% in the Czech Republic [31], 100% in Italy [2], and France [32]. However, some studies showed low-to-moderate isolation rates which ranged from 4% in Switzerland [5] to 13.5% in Australia [25], 33.6% in Belgium [10],

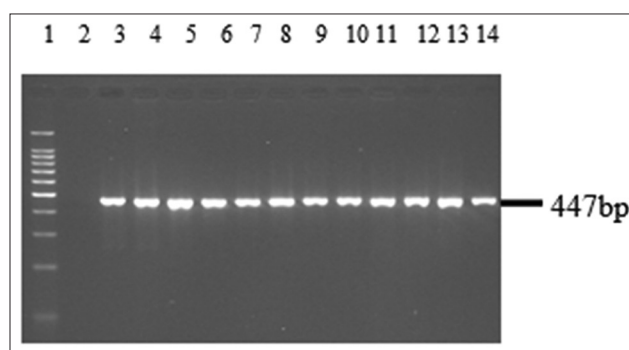


Figure-1: Modified species-specific polymerase chain reaction for *Helicobacter pullorum*. Lane: 1 - marker 100 bp ladder lane, 2 - *Campylobacter jejuni* ATCC 24928 lane, 3 - *H. pullorum* ATCC 51863 lane, 4-14 - *H. pullorum* isolates.

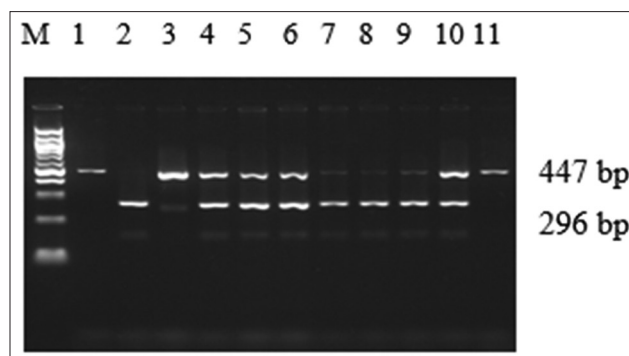


Figure-2: Modified polymerase chain reaction for *Helicobacter pullorum* and *Campylobacter* spp. Lane M - marker 100 bp ladder; lanes: 1 - *H. pullorum* ATCC 51863, 2 - *Campylobacter jejuni* ATCC 24928, 3 - *H. pullorum* CCUG 33837, 4-6 mixed isolates from fly samples, 7-9 mixed isolates from floor samples, 10 - mixed isolate from broiler samples, 11 - *H. pullorum* ATCC 51863.

Table-1: Occurrence of *H. pullorum* in broiler chickens in 10 farms.

Farms	Number of samples	Isolations		Number of positives (%)
		<i>H.p</i>	Mix*	
9	10	8	0	8 (80)
10	10	9	0	9 (90)
11	10	8	0	8 (80)
12	10	8	0	8 (80)
13	10	7	0	7 (70)
14	10	3	0	3 (30)
15	10	2	0	2 (20)
16	10	0	0	0 (0)
17	10	0	0	0 (0)
18	10	0	6	6 (60)
Total	100	45	6	51 (51)

H.p=*Helicobacter pullorum*; *Mix: Mixed culture consisting of *H. p* and *Campylobacter* spp. Farms 9-15 and 18 practiced open-house system; Farms 16 and 17 practiced closed-house system

and 39.3% in Egypt [8]. Manfreda *et al.* [33] found that chickens reared in free-range farms had lower occurrence (57%) compared to those reared in conventional (84%) and organic (97%) farms, warranted further investigation by the authors. In this study, the presence of the organisms in chickens was probably

Table-2: Occurrence of *H. pullorum* isolates in broiler chickens and environmental samples in 10 farms.

Farms	Chickens	Flies	Floor	Water
9	+	+	+	-
10	+	-	-	-
11	+	+	+	-
12	+	+	+	-
13	+	-	-	-
14	+	-	-	-
15	+	-	-	-
16	-	-	-	-
17	-	-	-	-
18	+	-	-	-

*Positive for *H. pullorum*, -negative for *H. pullorum*.
H. pullorum=*Helicobacter pullorum*

Table-3: Occurrence of *H. pullorum* in water, flies, and floor and soil.

Type of samples	Number of samples	Number of positive isolates (%)	
		<i>H.p</i>	Mix*
Water	40	0	0 (0.0)
Flies	40	0	7 (17.5)
Floors and soils	20	0	6 (30.0)

H.p: *Helicobacter pullorum*; *Mix: Mixed culture consisting of *H.p* and *Campylobacter* spp.

due to the unhygienic condition of housing facilities, high fly populations, presence of wild birds and rats around farm environment and in chicken houses, and lack of biosecurity measures such as absence of foot-bath and presence of water puddles under some of the chicken houses which were observed during the study. The presence of *Campylobacter* spp. together with *H. pullorum* was found on one farm. Shen *et al.* [29] had reported the simultaneous presence of *Helicobacter* spp. and *Campylobacter* spp. in cats.

It is interesting to note that *H. pullorum* was not isolated from chickens in two farms which practiced closed-housing system. It is most likely due to good hygiene and husbandry practices, and birds, as well as insects, had no access to the chicken houses. Furthermore, the environmental samples collected from these farms were also negative for *H. pullorum*. A similar finding was reported by Tang *et al.* [34] who did not isolate any *Campylobacter* in chickens in closed-housing system compared to 95% in chickens reared under open-housing system. Bull *et al.* [23] showed that the transmission of *Campylobacter* spp. can occur from puddles outside the facility to broiler flocks, feed, water, litter, and air within the house despite the implementation of standard biosecurity measures in modern broiler house.

In this study, *H. pullorum* was not present in the water samples. Azevedo *et al.* [12] reported that all *Helicobacter* spp. were sensitive to light and temperature (37°C) and all tested strains lost their culturability within 24 h of exposure. UV rays and higher temperatures (>42°C) have been shown to destroy and even kill *Campylobacter* cells [28]. The absence of *H.*

pullorum in water as well as in the environment may not only be due to a small number of *H. pullorum* in the sampled water but may also be due to presence of contaminating robust microorganisms or the organisms were in nonculturable but viable form could be inactivated due to warm temperature. In most of the open-house farms, the water tanks were not covered and readily exposed to warm temperature (37°C). The uncovered water tanks were exposed to high temperature and UV which may have contributed to the absence of viable *H. pullorum* in water and farm environments.

Flies from three farms that practiced open-housing system were found co-colonized with *H. pullorum* and *Campylobacter* spp. Rosef and Kapperub [16] isolated *Campylobacter* from 50.7% of flies in broiler farms, while Hald *et al.* [18] found an 8.2% carriage rate of *C. jejuni* in flies captured in ventilation inlets of broiler house. *H. pylori* in flies was reported to serve as a reservoir and a vector to chickens and man as well as the environment [13]. From this study, it may be possible that flies may have had a role as reservoirs in the spread *H. pullorum* and *Campylobacter* to chickens in the farms or on having in contact with the organisms from the chickens and/or the contaminated environment may have disseminated the organisms. Apart from flies, wild birds have also been reported to carry *Campylobacter* and *H. canadensis*, a probable zoonotic pathogen and closely related to *H. pullorum* [35]. Wild birds were observed around and in the chicken houses in the open-housing system. The floor samples in three open-house farms too were contaminated with *H. pullorum* and *Campylobacter* spp. It could be that the organisms shed in the feces had contaminated the floors and soils. The potential role of litter in the transmission of *Campylobacter* in poultry house from infected chicks was described by Montrose *et al.* [19]. Further study is recommended to investigate the role of environment in the spread of *H. pullorum*. The study also showed a high occurrence of *H. pullorum*, indicating that they may be a frequent intestinal colonizer of broiler chickens and thus may represent a health risk to humans.

Conclusion

This study provided further information on the occurrence and spread of *H. pullorum* in poultry. The closed-housing system showed that good biosecurity measures and good management and husbandry practices could minimize or control the presence of *H. pullorum* and *Campylobacter* spp. in broiler chickens and the farm environment; thus, the establishment of more of such farms is highly recommended.

Authors' Contributions

SA conceived and designed the study. SSW and JA collected the samples and SSW carried out the experiment. AAB, SSW, and ZZ analyzed the data. SSW wrote the first draft of the manuscript. SA and

AAB revised the manuscript. All authors read and approved the final draft of the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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