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Detection of *Salmonella* Spp. in Feed and Their Antibiotic Susceptibility for Alternative Therapy

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ABSTRACT

In the study, about 695 samples comprising (fish powder: 320 samples, blood meal: 41 samples, bone meal: 123 samples), finished feed (pellets of pig feed: 213 samples) were collected and detected by polymerase chain reaction (PCR). Isolation prevalence in fish powder, blood meal, bone meal, finished feed was 23(7.19 %), 9 (21.95%), 48 (39.67%), 2 (0.94%) respectively. These *Salmonella* showed different antibiotic sensitivities to erythromycin, ampicillin, penicillin, ciprofloxacin. However, all these strains were inhibited with plantaricin produced by *Lactobacillus plantarum* PN05 isolated in *Coryandrium sativum*. Our findings highlighted a potential public health hazard and warned human the outbreaks of human salmonellosis with high resistance due to the consumption of contaminated feed and also suggested the prevention by plantaricin of *Lactobacillus plantarum* PN05.

INTRODUCTION

Salmonella causes a health problem in the world. In United States, *Salmonella* infected in eggs was detected (Braden, 2006). *Salmonella* that occurred in developing countries commonly affect feed. Detection of *Salmonella* in feed is necessary in the processing chain guarantees. The identification, typing and fingerprinting of *Salmonella* were performed in the old days (Threlfall and Frost, 1990). Currently, polymerase chain reaction (PCR) is becoming the most utilized rapid method to detect *Salmonella* in food. In this context, several PCR-based assays have already been described (Iun-Fan *et al.*, 2008). However, only some of these assays are applicable as diagnostic tools. Although there are numerous alternative methods for *Salmonella* detection, their application in feed is still narrow because of the presence of PCR inhibitors in the sample. Moreover, antibiotic resistance in *Salmonella* is high, leading the difficulties in treatment of salmonellosis (Hald *et al.*, 2007). Rapid detection will contribute to the study on the risk of *Salmonella* to human because there was an association of phylogeny and virulence (Litrup *et al*., 2010).

Moreover, the antibiotic susceptibility of *Salmonella* would be exploited soon so that a treatment would be alternative (Hendriksen *et al.*, 2007). Nowadays, bacteriocins isolated from *Lactobacillus* are potential in pathogen prevention. However, each bacteriocin shows their significant effects on different pathogens. With the rapid detection, a rapid collection of pathogens obtained for solving many important problems caused by pathogens.

Therefore, the study established the polymerase chain reaction (PCR) method for *Salmonella* detection and then the antibiotic susceptibility was done. Then, an alternative therapy by bacteriocins of *Lactobacillus plantarum* was also tested.

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MATERIALS AND METHODS

Sample collection and bac-terial cultivation

The entire sample derived from analytical service at Center of analytical service and experimentation (CASE) in Hochiminh city. Three feed ingredients (fish powder: 320 samples, blood meal: 41 samples, bone meal: 123 samples), finished feed (pellets of pig feed: 213 samples). All the samples were cultured in Luria broth.

DNA extraction and PCR

DNA was extracted from above samples using a modified PowerPrep™ DNA extraction from food and feed kit (Kogenebiotech). One mL buffered peptone water (BPW) aliquot of each frozen sample was diluted with 400 µL of lysis buffer A and 40 µL of lysis buffer B in a microtube and mixed for 10 sec; 10 µL of proteinase K and 10 µL RNase A were added before incubation at 65 ^{o}C for 1 hour; then, 400 µL of chloroform was added and the samples were centrifuged for 15 min at 12,000 rpm. 200 µL of supernatant was used to extract the DNA according to the manufacturer's instructions. For the PCR assay, concentrations of reagents for PCR reaction were used according to AccuLite *Salmonella* spp. Detection Kit (KT Biotech) with a primer pair (F: 5'-TAC TTA ACA GTG CTC GTT TAC-3') and (R: 5'-ATA AAC TTC ATC GCA CCG TCA-3') (Iun-Fan *et al.*, 2008), targeting the *invA* gene of *Salmonella* spp. to obtain an amplicon with 570 base pairs. 5 µL sample was used for PCR. The total volume for PCR was 25 µL. The temperature program was 94 ^{o}C for 15 min in the first cycle. The conditions for next 40 cycles were 94 ^{o}C (30 s), 60 ^{o}C (30 s) and 70 ^{o}C (30 s). Then, one next cycle was 72 ^{o}C for 5 min. Finally, the reaction was cooled to 4 ^{o}C. The PCR products were analyzed with gel electrophoresis using 1 % agarose gels containing ethidium bromide (0.5 mg/mL) in TBE buffer (89 mM Tris-HCl pH 8.3, 89 mM boric acid, 2.5 mM EDTA). The DNA bands were observed by irradiating the pre – stained gel under UV illuminator at 302 nm and photographed.

Antibiotic susceptibility test

To test the antibiotic susceptibility of isolated *Salmonella*, minimum inhibition concentration (MIC) should be determined. In this study, the antibiotics (ampicillin, penicillin, erythromycin, ciprofloxaxin) were diluted from 1024 to 1 µg/mL in 1mL broth volume in standard test tubes. One inoculum of *Salmonella* (10^6 cfu) was mixed in test tubes and incubated in 12h. For the suspect tubes, they were checked for the bacterial survival on agar to make sure the definite inhibition. The tests were performed by triplicate. The lowest concentration of antibiotics in which microorganism was not survival is the minimal inhibitory concentrations.

Plantaricin preparation and its anti- *Salmonella* activity test

Lactobacillus plantarum PN05 isolated in *Coryandrum sativum* (Le *et al.*, 2015) was cultured in De Man-Rogosa-Sharpe (MRS) (Biokar Diagnostics, Beauvais, India) and incubated at 37 ^{o}C under aerobic conditions. Cultures were collected at different phase of incubation and centrifuged at 10000 rpm for 30 min to separate the cell from the broth. The cell-free supernatant was precipitated with 40% ammonium sulphate. The supernatant was precipitated continuously with 60% ammonium sulphate. The pellet was collected and solubilized in water. This solution was dialyzed in dialysis tube (cut-off: 1KDa) to eliminated ammonium sulphate. This final solution contained plantaricin. Plantaricin concentration was determined by spectrophometer. Plantaricin was used for its anti-*Salmonella* activity, using agar diffusion test according to Tagg (Tagg *et al.*, 1971). In this study, plantaricin were used 20 µg/mL to applied 6 mm wells on plates inoculated with one inoculum of *Salmonella* (10^6 cfu) and then incubated in 12h.The diameter of inhibition zones were measured next day. The tests were performed by triplicate.

Statistical analyses

The SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to calculate the means and standard deviations in any experiments involving triplicate analyses of any samples. The statistical significance of any observed difference was elauated by oneway analysis of variance (One way ANOVA). Difference at $P < 0.05$ were considered to be significant.

RESULTS

Detection of *Salmonella*

Salmonella detection was presented in table 1. The ratio of *Salmonella* spp. detected by the PCR method was 11.80%.

Table 1: Detection *Salmonella* spp. in the samples.

	n	%
Fish powder (320)	23	7.19
Blood meal (41)	9	21.95
Bone meal (121)	48	39.67
Finished feed (213)	2	0.94
Total (695)	82	**11.80**

The study showed that PCR could be applied to detect *Salmonella* spp. in feed. The results in table 1 also showed that feed had a high risk to *Salmonella* infection, especially the blood meal (21.95%) and bone meal (36.97%). Although there was a lower percent of *Salmonella* detected in fish powder (7.19%), it was meant that there was a big problem of fish sources and food processing.

Antibiotic susceptibility test

Among isolated *Salmonella* spp., 11 strains were used for antibiotic susceptibility test. The results were showed in table 2, 3, 4, 5, 6.

There were detected 2 strains (Sal 5 and Sal 8) showing ampicillin resistance of microorganisms (Table 2). Interestingly, Sal 7 showed strong resistance to penicillin (Table 3). For ciprofloxacin resistance, Sal 7 and Sal 10 showed strong resistance (Table 4). All 11 strains showed resistance to erythromycin (Table 5).

Table 2: The antibiotic sensitivity of strains with ampicillin.

	Ampicillin concentration (µg/mL)							
	1024	512	256	128	64	32	16	0.8
Sal 1	-	-	-	-	-	+	+	+
Sal 2	-	-	-	+	+	+	+	+
Sal 3	-	-	-	-	-	-	+	+
Sal 4	-	-	-	-	-	+	+	+
Sal 5	-	+	+	+	+	+	+	+
Sal 6	-	-	-	-	-	-	+	+
Sal 7	-	-	-	+	+	+	+	+
Sal 8	-	+	+	+	+	+	+	+
Sal 9	-	-	-	-	-	-	+	+
Sal 10	-	-	-	+	+	+	+	+
Sal 11	-	-	-	+	+	+	+	+

Data was in triplicates.

Table 3: The antibiotic sensitivity of strains with penicillin

	Penicillin concentration (µg/mL)							
	1024	512	256	128	64	32	16	0.8
Sal 1	-	-	+	+	+	+	+	+
Sal 2	-	-	-	-	-	+	+	+
Sal 3	-	-	-	-	-	-	+	+
Sal 4	-	+	+	+	+	+	+	+
Sal 5	-	-	-	+	+	+	+	+
Sal 6	-	-	+	+	+	+	+	+
Sal 7	+	+	+	+	+	+	+	+
Sal 8	-	-	+	+	+	+	+	+
Sal 9	-	-	+	+	+	+	+	+
Sal 10	-	-	-	+	+	+	+	+
Sal 11	-	-	+	+	+	+	+	+

Data was in triplicates.

Table 4: The antibiotic sensitivity of strains with ciprofloxacin

	Ciprofloxacin concentration (µg/mL)							
	1024	512	256	128	64	32	16	0.8
Sal 1	-	-	+	+	+	+	+	+
Sal 2	-	-	-	+	+	+	+	+
Sal 3	-	-	-	+	+	+	+	+
Sal 4	-	-	+	+	+	+	+	+
Sal 5	-	+	+	+	+	+	+	+
Sal 6	-	-	-	+	+	+	+	+
Sal 7	+	+	+	+	+	+	+	+
Sal 8	-	-	+	+	+	+	+	+
Sal 9	-	-	+	+	+	+	+	+
Sal 10	-	-	+	+	+	+	+	+
Sal 11	+	+	+	+	+	+	+	+

Data was in triplicates.

Table 5: The antibiotic sensitivity of strains with erythromycin.

	Erythromycin concentration (µg/mL)							
	1024	512	256	128	64	32	16	0.8
Sal 1	+	+	+	+	+	+	+	+
Sal 2	+	+	+	+	+	+	+	+
Sal 3	+	+	+	+	+	+	+	+
Sal 4	+	+	+	+	+	+	+	+
Sal 5	+	+	+	+	+	+	+	+
Sal 6	+	+	+	+	+	+	+	+
Sal 7	+	+	+	+	+	+	+	+
Sal 8	+	+	+	+	+	+	+	+
Sal 9	+	+	+	+	+	+	+	+
Sal 10	+	+	+	+	+	+	+	+
Sal 11	+	+	+	+	+	+	+	+

Data was in triplicates.

The antibiotic susceptibility tests pointed that there are many kinds of *Salmonella* showing the significant resistance to antibiotics when human contacts to their feed. Seriously, one hundred percent of *Salmonella* strains were resistant to erythromycin. It was meant that this is a warning that erythromycin should not be used for *Salmonella* treatment. Interestingly, these strains was strongly inhibited by plantaricin, a bacteriocin of *Lactobacillus plantarum* PN05 isolated in *Coryandrium sativum* (Table 1, Figure 1). Consequently, plantaricin can be used in preservation of food as well as in alternative treatment of *Salmonella*.

Table 6: The antibiotic inhibition of bacterocin.

	Inhibition zone (mm)
Sal 1	26±1.2
Sal 2	24±1.3
Sal 3	28±2.2
Sal 4	24±3.1
Sal 5	28±0.8
Sal 6	28±1.8
Sal 7	26±2.5
Sal 8	32±2.7
Sal 9	24±1.9
Sal 10	28±1.6
Sal 11	24±2.4

Data expressed by mean ±SD.

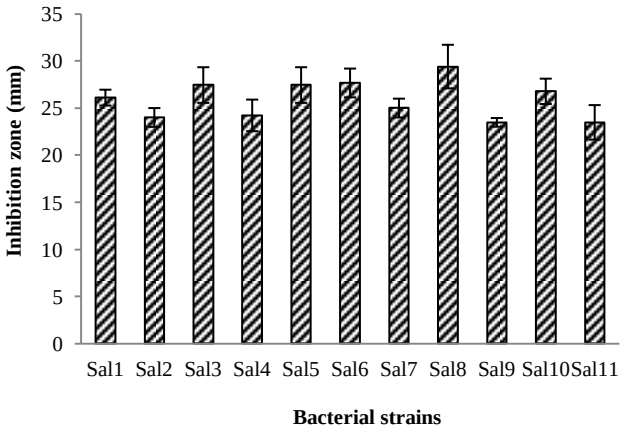

Fig.1: Inhibition zone of bacteriocin on Salmonella strains.

DISCUSSION

As seeing in table 1, the detection ability was high (11.8%) using PowerPrep™ DNA extraction from food and feed kit (Kogenebiotech), pointing the potency of this kit while there were many affordable DNA-extraction method from only *Salmonella enterica* for PCR experiments (Karimnasab *et al.*, 2013).

The resistance of *Salmonella* to amphicillin and penicillin due to *Salmonella* may contain beta-lactam gene (*bla*). However, there was the difference in resistance to ampicillin from penicillin in same *Salmonella* strains (Table 2 and 3), for example *Salmonella* strain (Sal 7). It was meant that there was the structural changes in penicillin binding protein in Sal 7, leading to resistance to penicillin not ampicillin.

As presented in table 4, Sal 7 and Sal 8 were resistant to ciprofloxacin due to multi-drug resistant (MDR) gene (Franco et al., 2015). Commonly, minimum inhibitory concentration (MIC) of *Salmonella* containing MDR was 0.25 µg/mL (Franco et al., 2015). The MICs of Sal 7 and Sal 8 were over 1024 µg/mL. The results in this study suggested Sal 7 and Sal 8 containing resistant markers other MDR. In Sal 1, Sal 2, Sal 3, Sal 4, Sal 5, Sal 6, Sal

9, Sal 10, Sal 11, the MICs were 128 µg/mL that was higher than 0.25 µg/mL, pointing that these strains probably contained resistant markers. Further study will be done to understand well the resistance of *Salmonella* isolated in feed.

Interestingly, all tested *Salmonella* strains (1-11) in table 5 were resistant to erythromycin that claimed us that *Salmonella* appearing in Vietnam was high resistant to this antibiotic. Therefore, using this antibiotic for *Salmonella* treatment should be checked carefully. These strains might have antibiotic efflux pump, leading low drug accumulation. However, the resistant mechanism should be more clarified.

Although *Salmonella* isolates were resistant to current antibiotics, *Salmonella* could be inhibited well with plantaricin of *Lactobacillus plantarum* AD1 (Table 6, Figure 1). It was meant that multi drug resistance (MDR) of *Salmonella* would not necessary to recognize plantaricin. The study indicated that plantaricin could be used in food preservation and alternative therapy for *Salmonella* infection.

With the diversity of *Salmonella* from different food sources due to a rapid, reliable PCR method, the information of drug susceptibility of *Salmonella*, the prevention in *Salmonella* infection will be effective. The factors relating to drug susceptibility will be announced soon.

CONCLUSION

The study supplied information for detection of *Salmonella* by PCR and the preliminary prevention of *Salmonella*. Moreover, the dug susceptibility of *Salmonella* isolated from feed warned us to use antibiotic carefully because of the high resistance in *Salmonella*.

Consequently, using this alternative antibiotic to treat *Salmonella* should have further research due to these strains might have antibiotic efflux pump, leading low drug accumulation. On the other hand, the resistant mechanism should be further explained.

Although *Salmonella* isolates were resistant to existing antibiotics, *Salmonella* could be inhibited acceptably with plantaricin of *Lactobacillus plantarum* AD1 as presented on this paper. It was expected that MRD of *Salmonella* recognize plantaricin. The results on this research indicate that plantaricin could be used in food preservation and alternative therapy for *Salmonella* infection.

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