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Salmonella Detection in Egg Powder Imported to Indonesia Through Port of Tanjung Priok, Jakarta and its Resistance Against Antibiotics

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Abstract: Egg powder becomes an alternative raw material instead the use of raw eggs in food production process. Some cases of microbial contamination on this product make it necessary to maintain a routine inspection to assure the safety of these product. The study was conducted to detect the presence of *Salmonella* in egg powder imported to Indonesia and its resistance to antibiotics. The study was performed using cross sectional study and the sample size was calculated based on assumption of confidence level of 95% with margin of error of 10% and predicted prevalence of 50%. Total of 100 egg powder samples was collected through August 2014 from two exporting countries, Ukraine (Whole egg powder, n=30) and India (Whole egg powder, n=40 and egg yolk powder, n=30). The study was performed by packaging inspection of the product followed by samples collection and testing for *Salmonella* contamination using rapid test and conventional isolation and identification methods. Isolated *Salmonella* were confirmed by PCR assay and then tested for antibiotic resistance. There were 5 samples containing *Salmonella. Salmonella* isolates showed resistance against 5 types of antibiotics, i.e., ampicillin, amoxicillin-clavulanic acid, oxacillin, cephalothin, cefoxitin and 100% of the isolates had resistance against minimum of 3 types of antibiotics. These conditions should be taken into consideration since antibiotic resistance in *Salmonella* would cause negative impacts on human, animal and environmental health.

Key words: Antibiotic Resistance · Egg Powder · *Salmonella*

INTRODUCTION

Egg powder becomes an alternative raw material instead the use of raw eggs in food production process. Some cases of microbial contamination on this product make it necessary to maintain a routine inspection to assure the safety of these product. *Salmonella* is listed as one of few bacteria that reported can still be isolated from egg powder.

The fact that *Salmonella* can still be found contaminating egg powder was traced back to 1942. *Salmonella* was being isolated from dried egg powder received in the British Isles for food purposes. *Salmonella* found on egg powder was identical to *Salmonella* that commonly isolated from animal, birds or human [1]. By the year 2011, *Salmonella* was isolated from Canadian produced egg powder. *Salmonella* was able to be isolated from 28 of 380 egg powder samples tested [2]. The most recent reports of *Salmonella* findings on egg powder were published by The U.S Department of Agriculture's Food Safety and Inspection Service (FSIS) on February of 2014. *Salmonella* contamination was found on egg powder during routine testing of egg powder produced by a company on Washington State [3].

The genus *Salmonella* belongs to the family Enterobacteriaceae. *Salmonella* is facultative anaerobic, Gram-negative, oxidase-negative and rod-shaped bacteria. The genus *Salmonella* consists of two species, *Salmonella enterica* and *S. bongori*. More than 2,400 serovars are known [4]. Historically *Salmonella* had been

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named based on the original places of isolation such as *Salmonella* ser. London and *Salmonella* ser. Indiana [4,5]. This nomenclature system was replaced by the classification based on the susceptibility of isolates to different selected bacteriophages which is also known as phage typing [5].

Salmonella serovars are widespread in nature and can be found in the intestinal tract of all animals species, both domestic and wild which result in a variety of *Salmonella* infection sources. Salmonellosis represents an important foodborne disease that continues to pose a major and unacceptable threat to human public health in both developed and developing countries [6].

The resistance of *Salmonella* to antibiotics is growing as a global concern. The frequency of *Salmonella* strains resistant to one or more antibiotics have increased and been reported in most part of the world. Another great concern is majority infections with multi drugs resistant *Salmonella* are acquired through the consumption of contaminated foods of animal origin such as swines and chicken and eggs [5].

The study was conducted to detect the presence of *Salmonella* in egg powder imported to Indonesia and to characterize its antibiotic resistance.

MATERIALS AND METHODS

Location and Time of Study: The study was performed on August to November 2014. The study was conducted by physical packaging inspection followed by sample collection and detection of *Salmonella* using commercially available rapid test kit and compared to standard isolation and identification methods for *Salmonella* detection. Presumed *Salmonella* isolates were confirmed by polymerase chain reaction (PCR) assay. *Salmonella* isolates obtained from the samples collected were then tested for antibiotic resistance. Physical packaging inspection and sample collection was performed on Animal Quarantine Inspection Facility of Port of Tanjung Priok, Jakarta, Indonesia. Laboratory works including rapid test, isolation and identification, PCR assay and antibiotic resistance test was performed on Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Bogor Agricultural University.

Study Design: The study was performed using cross sectional study and the sample size was calculated based on assumption of confidence level of 95% with margin of error of 10% and predicted prevalence of 50%. Sample collection was focused on importation that arrived on the Port of Tanjung Priok, Jakarta, Indonesia on August 2014. Samples was collected by simple random sampling methods. Samples collection was considering country of origin, type of egg powders and production batch. Positive and negative control was always included throughout laboratory examination performed on samples. Positive control used in this study was commercial *Salmonella typhimurium* isolate ATCC 14028. Negative control used in this study was commercial *Escherichia coli* isolate ATCC 25922.

Sample Collection: Total of 100 egg powder samples were collected through August 2014. Samples were collected from two exporting countries i.e., Ukraine (Whole egg powder, n=30) and India (Whole egg powder, n=40 and egg yolk powder, n=30). Samples were collected from seven different shipments. Each shipment involved different batch numbers and different types of egg powder treated as one sampling target and ten samples were collected. Sample collection were executed aseptically to ensure no cross contamination occurred during sample collection.

Rapid Test of *Salmonella*: Egg powder samples were tested using commercially available rapid test RIDA®COUNT *Salmonella*/Enterobacteriaceae (R-Biopharm AG, cat# R10100201). Samples used in this rapid test was previously pre-enriched at 37 ± 2°C for 18-20 hours in buffered peptone water (BPW) by diluting 25 g of sample into 225 ml BPW based on manufacturer recommendation. Test was performed by applying 1 ml of sample dilution after pre-enrichment period into rapid test dry medium plates. Dry medium plates then incubated for 24 hours at 35°C. Test reading was performed based on color formation on the surface of test kit.

Isolation and Identification of *Salmonella*: Isolation and identification performed on this study was based on methods described by Andrews *et al*. [7]. Modification to the methods was done on pre-enrichment phase which was replacement of lactose broth with BPW for sample dilution. Isolation and identification of *Salmonella* consisted of four phase. First was pre-enrichment phase where 25 g of egg powder sample was diluted into 225 ml of BPW and incubated 18 - 20 hours at 37°C. Next phase was selective enrichment where 1 ml of sample suspension from pre-enrichment phase were transferred into selective enrichment media Rappaport Vassiliadis (RV) broth. Selective enrichment in RV broth was incubated for 24 hours at 42°C. Third phase was plating

into selective media for *Salmonella* characterization. Selective media used in this study was xylose lysine deoxycholate agar (XLDA) and bismuth sulfite agar (BSA). Plate inoculation was performed by streaking enriched sample suspension into solid media of XLDA and BSA and then incubated for 24 hours at 37°C. *Salmonella* colonies were examined based on its characteristics in selective media used. Fourth phase, two or more suspect colonies from each selective plating medium were then inoculated into triple sugar iron agar (TSIA) and lysine iron agar (LIA) and then incubated at 37°C for 24 hours. Fourth phase also consist of testing of suspect *Salmonella* colonies for their biochemistry reaction patterns.

***Salmonella* Confirmatory Test by PCR:** Confirmation of *Salmonella* isolates were conducted by PCR assay using a 20-bp forward primer (5 - GGG GTG GAT TCT ACT CAA C – 3) and a 20-bp reverse primer (5 – AGA AGC GGA ACT GAA AGG C – 3) according to Woods *et al*. [8]. PCR was conducted using GeneAmp ® PCR System 9700 and was carried out in a total volume of 25 µl containing 1 µl (10 pmol/µl) of each primer, 12.5 µl Kapa Taq Extra HotStart, 5.5 µl dH_2O and 5 µl DNA template from samples.

An initial denaturation at 94°C for 5 min was followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 30 sec. Finally, an additional extension was achieved for 7 min at 72°C. Aliquot of PCR product was taken 10 µl each and electrophoresed on a 1.5% agarose gel for 1.5 h at 100 V, stained with ethidium bromide (0.5 µg ml^{-1}) and visualized and photographed under UV illumination.

Antibiotic Resistance Test of *Salmonella* Isolates: Antibiotic resistance test performed on *Salmonella* isolates was based on disc diffusion methods described by Clinical and Laboratory Standards Institute [9]. *Salmonella* isolates were grown on nutrient agar (NA) prior to testing procedure. After homogeny growth achieved, one loop of colony was transferred into tryptic soy broth (TSB) and incubated for 24 hours at 37°C until cloudy appearance of bacterial growth on the medium was visible. *Salmonella* suspension from TSB was then transferred into tube containing 9 ml of BPW gradually and homogenized to reach an equal turbidity of 0.5 Mc Farland suspension. After equal turbidity achieved, 0.1 ml of the *Salmonella* suspension inoculated into solid Mueller-Hinton agar (MHA) by spreading the suspension using hockey stick. Antibiotics discs and blank discs were then attached into the surface of inoculated MHA medium. Plate was then incubated for 24 hours at 37°C and antibiotics inhibition zone then measured. Justification of susceptible (S), Intermediate (I) and resistant (R) reaction was done based on the size of antibiotics inhibition zone measured [9].

There were 10 types from five groups of antibiotics used in this study. β-lactams antibiotics used in the testing was ampicilin 10 µg (AMP; OXOID CT0003B), amoxicillin-clavulanic acid 30 µg (AMC; OXOID CT0223B) dan oxacillin 5 µg (OX; OXOID CT0040B). Aminoglycosides antibiotics used in the testing was gentamicin 10 µg (CN; OXOID CT0024B) dan kanamycin 30 µg (K; OXOID CT0026B). Cephalosporins antibiotics used in this study was cephalothin 30 µg (KF; OXOID CT0010B), cefoxitin 30 µg (FOX; OXOID), cefotaxime 30 µg (CTX; OXOID CT0166B). Quinolones antibiotics used was nalidixic acid 30 µg (NA; OXOID CT0031B) and Tetracycline antibiotics was tetracycline 30 µg (T; OXOID CT0031B). Blank disc (OXOID CT0998B) was used in the testing as a negative control

Data Analysis: Data collected from physical packaging inspection, rapid test, isolation and identification, PCR assay and antibiotics resistance test were descriptively analyzed to describe *Salmonella* occurrence and its antibiotics resistance profile in egg powders imported to Indonesia through the Port of Tanjung Priok, Jakarta, Indonesia.

RESULT

Physical packaging inspection of imported egg powders showed that the products were in good packaging condition. Egg powder products imported to Indonesia were generally transported in two layers of packaging. Primary layer were a plastic bags and fixed by plastic strap or notched at the opening end. Primary layer was then inserted into secondary layer (Outer packaging) commonly a carton box or a multi layer paper and plastic bags. All the packaging of the products were intact while arriving in inspection site.

Rapid test examination on egg powder samples showed that there were no indications of *Salmonella* presence on the sample tested. The kit used in this study was showing positive reaction on control sample tested. The kit was also gave result on possibility of other Enterobacteriaceae presence in the samples.

Table 1: Isolation and identification results of egg powders sample based on type and origin of the samples

Country of Origin	Type of Egg Powder Tested	Number of Samples Tested	Positive of *Salmonella*
Ukraine	Whole egg powder	30	0
India	Whole egg powder	40	1 (2.5%)
	Egg Yolk Powder	30	4 (13.3%)
Total	100	5 (5%)	

Table 2: Antibiotic resistance test results with resistance reaction type

	Number of isolates given resistance reaction based on antibiotics type used*									
Isolates	AMP	AMC	OX	CN	K	KF	FOX	CTX	NA	T
Salmonella (n=5)	2	1	5	0	0	3	3	0	0	0

*AMP: ampicillin, AMC: amoxicillin-clavulanic acid, OX: oxacillin, CN: gentamicin, K: kanamycin, KF: cephalothin, FOX: cefoxitin, CTX: cefotaxime, NA: nalidixic acid, T: tetracycline.

Tabel 3: Antibiotic resistance test results with intermediate reaction type

	Number of isolates given resistance reaction based on antibiotics type used*									
Isolate	AMP	AMC	OX	CN	K	KF	FOX	CTX	NA	T
Salmonella (n=5)	0	0	0	0	0	1	0	0	1	0

*AMP: ampicillin, AMC: amoxicillin-clavulanic acid, OX: oxacillin, CN: gentamicin, K: kanamycin, KF: cephalothin, FOX: cefoxitin, CTX: cefotaxime, NA: nalidixic acid, T: tetracycline

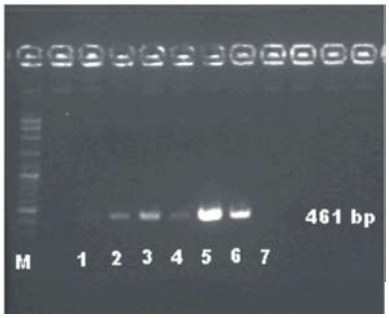

Fig. 1: Agarose gel electrophoresis of PCR products of *Salmonella* isolates

Lane M: 100 bp DNA marker (Vivantis, Vivantis Technologies), Lane 1 – 5: presumed *Salmonella* isolates, Lane 6: positive control, Lane 7: negative control.

Isolation and identification gave a different results that rapid test performed. Isolation and identification combined with biochemistry tests on presumed colonies showed there were *Salmonella* contamination on samples tested. Detailed results on *Salmonella* finding was described in Table 1.

There were no positive samples of *Salmonella* found on egg powder originated from Ukraine. The results was also showed that more contamination were found on egg yolk powder rather than in whole egg powder. Presumed *Salmonella* colonies were then confirmed by PCR assay. PCR assay result was showed that all presumed colonies were confirmed as *Salmonella* (Fig. 1).

Antibiotic resistance test performed to *Salmonella* isolates showed resistance reaction for some types of antibiotics tested. Detailed results of antibiotic resistance test with resistance reaction was described in Table 2.

Antibiotics resistance test results were not only showing resistance type reaction. The results also showed that there were isolate with intermediate reaction type on the test performed. Detailed results of antibiotic resistance test with intermediate reaction was described in Table 3.

Intermediate type results was one significance factor since it could become the potency of resistance reaction in the future. The more intermediate type result acquired, the more antibiotic resistance would occurs.

DISCUSSION

During the processing of egg powder, a pasteurization step was applied in the technology. There were two aspects must be taken into consideration. First was the destruction of as many contaminating microorganisms as possible. The second point was preventing the valuable components of the egg, mainly protein to break up [10]. Pasteurization protocols through egg powder production process were vary among producers, but considering the two above aspects, there will be less difference applied. Generally it takes through three main process. Pasteurization at 60°C for up to 6 minutes, continued to dehydration process using spray dried methods with inlet set to 121 - 145°C and outlet

55 - 60°C. Final phase was storage in hot storeroom with temperature of up to 70°C [10,11]. Hygienic control measurements show that the number of microorganisms in pasteurized liquid eggs can be up to 10^2-10^3 CFU/ml and sometimes *Salmonella* sp. can be found among the survivors [10]. This probably the main reason that *Salmonella* could still be found in the egg powder tested in this study. Another possible explanation would be contamination of *Salmonella* on the raw material or cross contamination during processing. *Salmonella* have been detected in unwashed eggs entering shell egg and egg product processing facilities [12].

Microbiological quality of produced egg powder will be highly determined by pasteurization of raw material and good sanitary controls throughout production line. Whole egg powder and egg yolk powder were commonly went through pasteurization process while it still in liquid form before proceeded into drying process. The combination between pasteurization and heat application while drying process resulted in extremely low bacterial contamination in the production of egg powder [13].

Confirmation of *Salmonella* findings in this study was carried out by PCR assay. PCR assay performed in this study was based on the use of primer from *Salmonella* origin of replication (*oriC*) region. The *Salmonella oriC* was chosen as a genus-specific region for an internal amplified control (IAC) to establish the specificity and sensitivity of the performed test. The *oriC* primer pair had been validated and amplified an expected 461-bp PCR product in all *Salmonella* strains tested and no product was obtained with the negative controls [8].

Salmonella became a major cause of gastroenteritis in human [4]. Water and food contaminated with fecal materials of animal or human carrier were the source of *Salmonella* infection. Cross contamination could happen through people handling the food, processing, tools contamination, or contamination during storage [14]. Salmonellosis was commonly attributed to table eggs with cracked or dirty shells or to egg products that had not been heated sufficiently during processing. Later identified that salmonellosis can spread through clean and intact high quality eggs [15]. *Salmonella* could infect eggs through two main route, vertically and horizontally [5,16].

Most studies have indicated that proper food handling and personal hygiene were the critical improvement areas to protect the public. Fecal contamination of food from human handling, cross contamination, time and temperature procedure failures have been shown to be the major sources of pathogenic infection. Other studies had specifically linked poor time and temperature processes and controls to food borne illness outbreaks [17].

Antibiotic resistance test results showed that *Salmonella* isolate generally resistance to β-lactam antibiotics. All of the isolates showed resistance to oxacilline. Oxacillin is first generation of penicillin with additional feature of resistance to β-lactamase enzyme produced by several bacteria. This type of antibiotics prescribed for treatment of infection caused by bacteria without the capability to produce β-lactamase enzyme [18,19]. *Salmonella* resistance to oxacillin correlated with its lipophilic characteristics making it hard to penetrate *Salmonella* cell wall [19].

Vast growing of intensified use of antibiotics in livestock rise a new potential problems with huge impact. Many study had indicated the occurrence of antibiotic resistance on *Salmonella* isolated from human and animals. Antibiotic resistance on *Salmonella* will limit the option of antibiotics given on therapy of salmonellosis. The limitation would not only affect treatment of salmonellosis in animals but also limit the ability to deal with *Salmonella* infection in human. Zoonotic infection of *Salmonella* would be worsened by this bacteria ability to survive from commonly use antibiotics during treatment [20].

CONCLUSION

There are *Salmonella* found on egg powder imported to Indonesia. The prevalence of *Salmonella* infection was 5% in this study. Isolated *Salmonella* showed resistance to several of antibiotics tested. Its show resistance reaction to five type of antibiotics tested i.e., ampicillin, amoxicillin-clavulanic acid, oxacillin, cephalothin, cefoxitin. All of the isolate had resistance reaction to minimum of three type of antibiotics tested. *Salmonella* isolates was showing intermediate reaction to two type of antibiotics tested i.e., cephalothine and nalidixic-acid. Intermediate reaction was showed to minimum of one type of antibiotic tested.

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