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Multidrug resistance among different serotypes of *Salmonella* isolates from fresh products in Indonesia

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Abstract: A total of 125 samples, consisting of 40 samples of chicken cuts, 30 samples of beef cuts and minced beef, 29 samples of fish, and 26 samples of vegetables were examined for aerobic plate counts and the presence of *Salmonella*. The samples were collected from the open market and from a supermarket in Bogor Indonesia. Based on the total plate counts, 35.2% of the fresh products showed a good to average quality. *Salmonellae* were detected in 24.8% of the samples examined. Chicken cuts were found as the most contaminated (52.5%), followed by beef (16.7%), fish (10.3%) and vegetables (7.7%). Serotyping of the isolates identified four serotypes: *Salmonella* Weltevreden*, S.* Kentucky*, S.* Typhimurium and *S.* Paratyphi C. Most of the isolates (n=15) exhibited resistance to erythromycin. Only one isolate of *S.* Kentucky, isolated from chicken cuts, showed intermediate resistance to chloramphenicol. Ten isolates showed resistance to at least two antibiotics. One strain of *S.* Weltevreden isolated from beef cuts demonstrated resistance to four antimicrobial agents (erythromycin, tetracycline, sulfamethoxazole, and streptomycin).

Keywords: *Salmonella*, fresh product, multidrugs resistance

Introduction

Acute gastroenteritis caused by *Salmonella* spp. remains to be a worldwide public health concern with the predominant serotypes from clinical cases varying with the geographical region. *Salmonella enterica* serovar Enteritidis is the most common in Europe, Central and South America, while *Salmonella enterica* serovar Typhimurium is predominant in Oceania, North America and Africa (WHO, 2006). Typhoid or paratyphoid fever, caused by *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi, continues to be endemic in many areas of the developing world, with the highest incidence reported in South-central Asia and South East Asia reaching over 100 per 100 000 cases/year (Crump *et al*., 2004). Published figures for Indonesia estimated a prevalence of 358 to 810 per 100.000 cases in 2007, with cases occurring throughout the year but peaking in the dry season (Hatta and Ratnawati 2008). However, the real magnitude of the problem is difficult to determine. In rural areas where access to diagnostic facilities is limited, there are probably many cases that remain undiagnosed.

A variety of foods have been implicated as vehicles transmitting salmonellosis to humans. Poultry, egg, and meat products have been continuously found as the most common food vehicles of the infection (Van Nierop *et al*., 2005; Suresh *et al*., 2006; Mrema *et al*., 2006; Little *et al*., 2008; Soltan-Dallal *et al*., 2009). However, reports on spreading of *Salmonella* from fish (Kumar *et al*., 2003) as well as reports indicating that raw vegetables may harbor potential *Salmonella* (Salleh *et al*., 2003; Abadias *et al*., 2008) are increasing. Contamination can occur along the food chain including production, processing, distribution, retail marketing, and handling/preparation.

The spreading of multidrug-resistant phenotypes has also been increasingly described among Salmonella serovars worldwide in many reports (Zhao *et al*., 2003; Ponce *et al*., 2008). A contributing factor in the development of resistance stems from the use of antimicrobials in human medicine, veterinary medicine, animal husbandry, as well as agricultural and aquaculture practices (Zhao *et al*., 2003). These routine practices are important factors in the emergence of antibiotic-resistant bacteria that subsequently can be transferred to humans through the food chain.

Reports of *Salmonella* contamination on food in Indonesia are limited. This bacterium was identified in Indonesia as the third important pathogen causing diarrhea after vibrios and shigellas (Tjaniadi *et al*., 2003). However, it has been expected that *Salmonella* were more spread by food, whereas *Vibrio* and *Shigell*a were particularly spread through water. Vollaard *et al*. (2004) reported that typhoid fever in Jakarta Indonesia is spread predominantly within the household, whereas paratyphoid is mainly transmitted outside the home. Since in paratyphoid fever, in

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some cases, a higher dose of bacteria is required for infection than in typhoid fever, consequently, food is implicated as the major vehicle for transmission of paratyphoid fever because *Salmonella* can multiply in food.

Fresh foods in Indonesia are typically sold at two types of markets i.e. supermarkets and open markets. The supermarkets, which are indoor markets, often display prepackaged or fresh products under refrigeration. In contrast, the open markets usually display unwrapped products at ambient temperatures. The present investigation was carried out to study the occurrence and antimicrobial resistance pattern of Salmonella from fresh foods i.e. chicken cuts, meat, fish, and vegetables collected from open markets and supermarkets in Bogor Indonesia. The total aerobic counts were also determined to evaluate the microbiological quality of the products.

Materials and Methods

Sample collection and microbial analysis

Chicken cuts (40 samples), beef cuts (20 samples), minced meat (10 samples), fish (29 samples), and vegetables (26 samples) were collected from various open markets and supermarkets in Bogor, Indonesia. The fish selected were white promfet (*Pampus argenteus*, 10 samples), gouramy (*Osphronemus gouramy*, 9 samples) and red tail scad (*Decapterus* Tabl. 10 samples). The vegetables selected were mungbean sprout (tauge, 10 samples), whole poh-pohan leaf (*Pilea trinervia*, 10 samples), and whole Chinese lettuce (*Lactuca sativa*, 10 samples). The samples were transported in a cool box with ice to the laboratory, and analyzed at the same day of the sample collection..

Microbial determinations were carried out using the standard methodologies described in the Bacteriological Analytical Manual (Maturin and Peeler, 2001). Twenty-five grams of each sample were diluted in 225 ml of Butterfield's phosphate-buffered dilution and homogenized for 2 minutes at normal speed in a Stomacher® (type 400 Circulator, Seward, Laboratory blender, England). Serial dilutions of the suspension were made in Butterfield's phosphate-buffered and analyzed for aerobic plate count.

Isolation and confirmation of Salmonella *serovars*

Isolation and confirmation of *Salmonella* were conducted using the methodologies described in the Bacteriological Analytical Manual (Andrews and Hammack, 2007). Twenty five grams of samples were weighed aseptically into sterile stomacher bags containing 225 mL of Lactose broth (Oxoid, Basingstoke, England) and subsequently homogenized in a Stomacher® (type 400 Circulator, Seward, Laboratory blender, England) for 2 minutes. The pH was determined using test paper after well mixing by swirling, and was adjusted if necessary to 6.8 ± 0.2. All homogenates were incubated 24 ± 2 h at 35°C for the pre-enrichment. After incubation, 0.1 mL of homogenate was transferred to 10 mL of Rappaport-Vassiliadis broth (RV, Oxoid) and 1mL to 10 mL of Tetrathionate Brilliant Green broth (TT, Difco) which were then incubated for 24 ± 2 h at 42 ± 0.2°C. A loop full of each broth was then streaked onto Bismuth Sulfite Agar (BSA, Oxoid), Hektoen Enteric Agar (HEA, Oxoid) and Xylose Lysine Desoxycholate Agar (XLDA, Oxoid). These plates were incubated for 24 ± 2 h at 35 ± 2.0°C.

After 24 ± 2 h incubation, two presumptive *Salmonella* colonies were picked from each selective agar and for confirmation inoculated in Triple Sugar Iron agar (TSIA, Difco) and Lysine Iron Agar (LIA, Oxoid). The TSIA and LIA slants were then incubated at 35± 0.2°C for 24 ± 2 h and examined for the reactions characteristic for suspected *Salmonella*. In TSIA, *Salmonella* typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar). In LIA, *Salmonella* typically produces alkaline (purple) reaction in butt of tube. Only distinct yellow in butt of tube was considered as acidic (negative) reaction. Most *Salmonella* cultures produce H_2S in LIA (Andrews and Hammack 2007).

From each combination of presumptive -positive TSIA and LIA slants material was inoculated in urea broth (Oxoid). If the reaction on urea was negative, strains were maintained on tryptone soya agar (TSA, Merck). Cultures were further identified with API 20E (Biomérieux, France) and serological tests using polyvalent 'O' and 'H' antisera.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS 2000 a,b) using the disk diffusion technique with commercially available discs (Oxoid). The antimicrobials and concentrations in micrograms tested were erythromycin 30 (Ery), tetracycline 30 (Tet), sulfamethoxazole 25 (Smx), chloramphenicol 30 (Chl), and streptomycin 10 (Str). The inhibition zones, in mm, were measured in duplicate and scored as sensitive, intermediate susceptibility and resistant according to the NCCLS recommendations, e.g. >19, 15-18 and < 14 for tetracycline; >18, 13-17 and <12 for chloramphenicol; etcetera (NCCLS 2000 a).

Statistical analysis

The univariate analysis of variance (SPSS for Windows 2003/2007 Release 13) was used to assess differences ($p \leq 0.05$) between aerobic plate counts of the different group. Furthermore, paired-sample t-test procedure was used to assess differences ($p \leq 0.05$) between aerobic plate counts of samples from open market and supermarket of the different group.

Results

In general, the highest plate counts were found in vegetables in comparison to those found in other fresh products. The aerobic plate count ranged from 6.2 to 8.5 $\log_{10}$ CFU/g in chicken cuts, from 4.4 to 8.4 in beef cuts, from 4.5 to 7.6 in fish and from 6.7 to 9.2 in vegetables (Table 1). Although the fresh products sold in supermarket were displayed at low temperature (10 ± 3ºC) while in open market were displayed at room temperature (28 ± 2ºC), microorganism counts in whole vegetables, however, showed no significant differences. Meat and fish sold in supermarket showed significant lower counts in comparison with that which were sold in open market; i.e. chicken cuts ($p = 0.00$), beef cuts ($p = 0.00$), red tail scad ($p=0.03$) and gouramy ($p = 0.01$).

Table 1. Total aerobic plate count (APC) and distribution of origin of the samples (n=125)

Sample	Origin	n	Range of APC ($\log_{10}$ CFU/g)	Average of APC ($\log_{10}$ CFU/g)[i)]
Chicken cuts	Open market	32	7.4 - 8.5	7.98^{a}
	Supermarket	8	6.2 - 7.4	6.59^{cdef}
Beef cuts	Open market	10	6.7 - 8.4	7.49^{abc}
	Supermarket	10	4.4 - 7.0	5.89^{fgh}
Minced beef	Supermarket	10	4.8 - 7.2	6.29^{efgh}
Red tail scad	Open market	3	6.5 - 7.4	6.79^{bcde}
	Supermarket	7	4.6 - 6.2	5.54^{h}
White promfet	Open market	3	6.1 - 7.6	7.01^{bcde}
	Supermarket	7	6.4 - 7.4	6.47^{defg}
Gouramy	Open market	2	6.6 - 7.3	6.97^{bcde}
	Supermarket	7	4.5 - 6.6	5.65^{gh}
Lettuce	Open market	5	6.8 - 8.0	7.34^{abcd}
	Supermarket	4	6.9 - 7.9	7.34^{abcd}
Mungbean sprout	Open market	4	6.7 - 9.2	7.67^{ab}
	Supermarket	4	7.1 - 9.2	7.96^{a}
Pilea	Open market	5	6.9 - 7.7	7.16^{abcde}
	Supermarket	4	6.9 - 7.4	7.17^{abcde}

[i)] Mean values ($\log_{10}$ CFU/g) that are not followed by the same letter are significantly different ($p < 0.05$)

The numbers of positive samples for *Salmonella* isolated from the fresh products are summarized in Table 2. Thirty one of 125 samples were contaminated with *Salmonella*, with chicken as the most contaminated (52.5%), followed by beef (beef cuts and minced beef, 16.7%). Relatively low *Salmonella* incidence in vegetables (7.7%) and fish (10.3%) was found in this study.

Table 2. Number of *Salmonella* positives samples from fresh products (n=125)

Sample	Total number of samples	Number of positive samples (%)		
		Open market	Supermarket	Total
Chicken cuts	40	17/32 (52.5)	4/8 (50.0)	21/40 (52.5)
Beef cuts	20	1/10 (10.0)	2/10 (20.0)	3/20 (15.0)
Minced beef	10	NA	2/10 (20.0)	2/10 (20.0)
Fish	29	1/8 (12.5)	2/21 (9.5)	3/29 (10.3)
Vegetable	26	1/14 (7.1)	1/12 (8.3)	2/26 (7.7)
Total	125	20/64 (31.3)	11/61 (18.0)	

NA: Not analyzed

Furthermore, serotyping was conducted for twenty (20) of thirty one (310 isolates originated from chicken cuts (10 of 21 isolates), beef (5 isolates), fish (3 isolates) and vegetable (2 isolates). Four different serovars of *Salmonella* were identified from the 20 isolates and are shown in Table 2. *S.* Weltevreden was the predominant serotype which accounted for 35% of the positive isolates, followed by *S.* Kentucky (30%), *S.* Typhimurium (25%) and *S.* Paratyphi C (10%). It was noted that *S.* Kentucky was present only in chicken cuts.

In this investigation, only one strain isolated from chicken cut from open market showed intermediate resistance to chloramphenicol, one of the drugs of choice in the treatment of human systemic salmonellosis. The other serovars were sensitive to this drug. Most of the isolates (n=15, 75%), however, exhibited resistance to erythromycin.

One strain of *S.* Weltevreden which was isolated from open market beef cuts, was resistant to four antimicrobials (erythromycin, tetracycline, sulfamethoxazole, and streptomycin). Two isolates were resistant to three antimicrobials, and 7 isolates were resistant to two antimicrobial tested.

Discussion

The results of this study indicated that the beef cuts, red tail scad and gouramy from supermarkets were in a good quality. The averages of the total aerobic counts of these samples were below the maximum limits of the Indonesian standard (National Standardization Agency, 2009), i.e. 6 $\log_{10}$ CFU/g for fresh chicken or beef and 5.7 $\log_{10}$ CFU/g for fresh fish. Most of chicken, beef and fish samples, in particular that sold in open market, exceeded the maximum limits. In this standard, however, the maximum limits of the total aerobic counts in raw vegetables are not regulated. However, the fact that the aerobic counts were more than 6 $\log_{10}$ CFU/g reflecting the exposure of the sample to any contamination and in

Table 3. *Salmonella* serovars isolated from fresh product

Origin	Samples	Serovar			
		***Salmonella* Weltevreden**	***Salmonella* Kentucky**	***Salmonella* Typhimurium**	***Salmonella* Paratyphi C**
Open market	Chicken cuts	-	5	2	1
	Beef cuts	1	-	-	-
	Fish	-	-	-	1
	Vegetable	1	-	-	-
Super market	Chicken cuts	-	1	1	-
	Beef cuts	2	-	-	-
	Minced beef	2	-	-	-
	Fish	-	-	2	-
	Vegetable	1	-	-	-
Total		**7 (35%)**	**6 (30%)**	**5 (25%)**	**2 (10%)**

Table 4. Resistance pattern Salmonella isolates

Isolate ID	Serotype	Resistance[a]	Product involved	Origin
PGB1	*S.* Typhimurium	Ery	Chicken cuts	Open market
PGB3	*S.* Typhimurium	Ery, Tet, Smx	Chicken cuts	Open market
S006	*S.* Typhimurium	Ery, Str	Chicken cuts	Supermarket
I149	*S.* Typhimurium	Ery	Red tail scad	Supermarket
PL2	*S.* Paratyphi C	Ery, Str	Chicken cut	Open market
I166	*S.* Paratyphi C	Ery, Str	Gouramy	Open market
PB5	*S.* Kentucky	Ery	Chicken cuts	Open market
PGB5	*S.* Kentucky	Ery, Smx, Str	Chicken cuts	Open market
PM3	*S.* Kentucky	Ery, Str	Chicken cuts	Open market
PKS1	*S.* Kentucky	Ery, Str	Chicken cuts	Open market
S3	*S.* Kentucky	Ery, Str	Chicken cuts	Supermarket
SP6	*S.* Weltevreden	Ery	Beef cuts	Supermarket
T4P2	*S.* Weltevreden	Ery, Tet, Smx, Str	Beef cuts	Open market
SG8	*S.* Weltevreden	Ery	Minced meat	Supermarket
VPBTM	*S.* Weltevreden	Smx	Vegetable	Supermarket
VTPB	*S.* Weltevreden	Ery, Str	Vegetable	Supermarket

[a] Smx, sulfamethoxazole; Str, streptomycin; Tet, tetracycline; Chl, chloramphenicol; Ery, Erythromycin

general, the existence of favorable conditions for the multiplication of microorganisms (Halablab *et al*., 2011).Furthermore, Technical Guidelines of Hazard Analysis and Critical Control Points - Total Quality Management (HACCP-TQM) lay down the microbial quality for raw foods, where the food containing aerobic plate count less than 4, 4-6.69, 6.69-7.69 and greater than 7.69 $\log_{10}$ CFU/g are rated as good, average, poor and spoiled food, respectively (Aycicek et al., 2006). Based on these criteria, the data of the present study indicated that 24.8% of samples could then be regarded as spoiled vegetable food, while 35.2% regarded as good to average quality.

The results of this study also highlighted the potential problem of spread of *Salmonella* serotypes, particularly in chicken cuts. The prevalence of *Salmonella* in fresh vegetables is considered low. However, it should not be underestimated, particularly in those eaten raw or lightly cooked, such as lettuce and pilea

Four different serovars of *Salmonella* were identified from the 20 isolates with the order from the most predominant i.e. *S.* Weltevreden, *S.* Kentucky, *S.* Typhimurium and *S.* Paratyphi C. The predominance of *S.* Weltevreden has been described in different reports among the Southeast Asian countries. WHO reported that *S.* Weltevreden was one of the important human *Salmonella* serovar after *S.* Enteritidis in Asia, which represented 10% and 33%, respectively (WHO, 2006). This serovar has been reported as a frequent and increasing cause of human infection in Thailand (Bangtrakulnonth *et al*., 2004) and Vietnam (Phan *et al*., 2005). *S.* Weltevreden was also found as the predominant serotype which accounted for 23.5% of the positive isolates isolated from the vegetables in Selangor Malaysia (Salleh *et al*. 2003). Furthermore, *S.* Weltevreden was also the most common serovar among the 210 isolates from imported seafoods from 20 countries during a 5-year period (2000–2005) (Ponce *et al*., 2008).

The presence of *S.* Typhimurium in chicken cuts and fish were recognized in this investigation. This serovar was also found in fish and crustaceans from various fish markets of Coimbatore South India as reported by Hatha and Lakshmanaperumalsamy (1997). More recent reports, however, indicated that *S.* Typhimurium was only found in pork among the meat analyzed (Padungtod and Kaneene, 2006; Duffy *et al*., 1999). Although *S.* Typhimurium is the most common *Salmonella* serovars causing Salmonellosis after *S.* Enteritidis worldwide (WHO 2006), but in Indonesia *S.* Typhi and *S.* Paratyphi A have been found to be more predominating serotypes (Tjaniadi *et al*., 2003).

Furthermore, this study notified that *S.* Kentucky was only present in chicken cuts. *S.* Kentucky was found before in chicken eggs (5.6%, n=54) in Bogor Indonesia (Rumawas *et al*., 1993). Reasons for differences in the distribution of *Salmonella* serotypes are not clearly understood (Duffy *et al*.,

1999), it may be attributed to seasonal or demographic factors. Variations in *Salmonella* prevalence may be attributed to several factors including the initial numbers of *Salmonella* in poultry, sanitation during slaughtering and processing of the poultry, possible cross-contamination at retail level and variations in the isolation methods conducted to detect the pathogen in individual studies.

This study also reported contamination of *S.* Paratyphi C in chicken cuts and fish from open market. *S.* Paratyphi C (causing paratyphoid fever), like *S.* Typhi (causing typhoid fever), is adapted to humans. However, *S.* Paratyphi C also occasionally infects animals (Liu *et al*., 2007). The potential source of *Salmonella* contamination is likely due to poor water quality, fecal contamination, poor sanitary conditions, or poor distribution and handling practices (Zhao *et al*., 2003).

This study showed that one strain of *S.* Weltevreden which was isolated from beef cuts was resistant against four antimicrobials (erythromycin, tetracycline, sulfamethoxazole, and streptomycin). Additionally, 9 isolates were resistant at least against two antimicrobial tested. Chloramphenicol, ampicillin, tetracycline and trimethoprim/sulfonamide combinations were the first antimicrobials used for the treatment of *Salmonella* infections in humans (Tjaniadi *et al*., 2003; Hatta and Ratnawati, 2009). Erythromycin is most often used for treatment of *Campylobacter* infections in Indonesia, while for cholera, tetracycline is used (Tjaniadi *et al*., 2003).

Multiple antibiotic resistant enteric pathogens have been reported in many developing countries, especially Pakistan, India, Bangladesh, and The Philippines (Rowe *et al*., 1997). Report from Indonesia indicate that trends in incidence of typhoid fever in Indonesia and the proportion of cases of typhoid fever attributed to multidrug resistance have gradually increased each year since 2001 (Hatta and Ratnawati, 2008). This report indicate that the levels of antibiotic resistance in *S.* Typhi from clinical cases in South Sulawesi has been rising and in 2007 6.8% of isolates were resistant to all three first line drugs, i.e. ampicillin, chloramphenicol and co-trimoxazole.

Furthermore, Thong *et al*. (2002) examined 95 strains of *S.* Weltevreden from different sources in Malaysia and found that the clinical isolates remained drug sensitive, but the vegetable isolates were resistant to at least two antibiotics. Aarestrup *et al*. (2003) found that 9.5% of the 503 *S.* Weltevreden isolates, from 10 countries, were resistant to one or more antimicrobial agents.

The use of antimicrobial agents in the treatment of diarrhea has greatly improved the quality of life among people in developing countries. In Indonesia, most hospitals and clinics treat diarrheal infected patients with antibiotics prior to receiving definitive laboratory results (Tjaniadi *et al*., 2003). However, since increased application of antimicrobial agents in both veterinary and human medicine is believed to be largely responsible for the emergence of drug resistance bacteria, the problems associated with microbial resistance will continue to pose a challenge to public health. Surveillance of antimicrobial resistance is essential for providing information on the magnitude and trends in resistance and for monitoring the effect of interventions, especially because the prevalence of resistance varies widely between and within countries, and over time (WHO, 2000). Particularly in developing countries, a concerted effort is needed by the medical services to implement reliable diagnosis in order to reduce misusing of antimicrobial agents in human medicine (Hatta and Ratnawati, 2008). It is also essential that antimicrobials be appropriately used in food animals, including aquaculture, on a global basis to preserve the efficacy of existing drugs and to limit the risk of transfer of resistant foodborne pathogens to humans (Zhao *et al*., 2003).

Conclusion

Based on the total counts, 35.2% of the fresh products (n=125) showed a good to average quality. *S.* Weltevreden was the predominant serotype found in the fresh products analyzed, followed by *S.* Kentucky, *S.* Typhimurium and *S.* Paratyphi C. Most of the *Salmonella* isolates were resistance to erythromycin, and ten isolates showed resistance to at least two antibiotics. The current study highlights the necessity for continuous monitoring of *Salmonella* in fresh products and its resistance to antimicrobial agents.

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