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A comparison of standard cultural methods for the detection of foodborne *Salmonella* species including three new chromogenic plating media

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Abstract

In this study the draft of the horizontal method for the detection of *Salmonella* species from human food and animal feed (ISO 6579:2002) was compared to the European gold standard (DIN EN 12824:1998), including the three new chromogenic plating media AES *Salmonella* Agar Plate (ASAP), Oxoid *Salmonella* Chromogen Media (OSCM) and Miller–Mallinson agar (MM). First the growth and appearance of 36 bacterial type strains (*Salmonella* and other 21 species) on ASAP, OSCM and MM were compared to those on the three traditional agars Brilliant Green Agar according to Edel and Kampelmacher (BGA), Xylose Lysine Deoxycholate Agar (XLD) and Xylose Lysine Tergitol 4 Agar (XLT4). Only on MM agar, did all of 36 tested type strains produce typical colonies, especially strains of *S.* Senftenberg, *Salmonella arizonae*, *S.* Dublin and *S.* Derby. Artificial inoculation experiments using raw pork ground meat ($n=92$) were subsequently conducted. A shortened incubation time of 24 h in RVS broth yielded a *Salmonella* species recovery of 100% from spiked meat samples. Finally, 286 naturally contaminated raw porcine and bovine minced meat samples and raw poultry meat samples were investigated. Forty-three strains from a total of 39 *Salmonella*-positive samples were found.

S. Typhimurium ($n=21$), with DT 104 L, DT 012 and RDNC being the most prevalent subtypes isolated. D-tartrate-positive *S.* Paratyphi B ($n=2$) and *S.* Saint-Paul ($n=3$) were also recovered. They were cultured from poultry meat and were multi-resistant against antibiotics including nalidixic acid.

Rappaport Vassiliadis broth with soypeptone (RVS) yielded the highest recovery of *Salmonella* spp. (97,4%) compared to Tetrathionate broth with Novobiocin according to Muller and Kauffman (MKTTn, 94,9%) and Selenite Cystine broth (SC, 38,5%). However, no significant difference was obtained by comparing the ISO 6579:2002 draft to the gold standard.
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1. Introduction

Infections with non-typhoid *Salmonella* are one of the most commonly recorded cause of gastroenteritis in humans in the western industrial countries. The great majority of human cases of salmonellosis are due to the consumption of contaminated foods of animal origin.

Enormous efforts in the areas of human and animal disease control as well as food hygiene has resulted in a visible reduction of food borne salmonellosis worldwide. Nevertheless, *Salmonella* will still be a risk to human health in the future (Anonymous, 2005, 2006).

The gold standard for the detection of *Salmonella* from food relies on a nonselective preenrichment, followed by a selective enrichment step, isolation on selective agar media and a preliminary biochemical and serological confirmation. This conventional cultural method is very time consuming and expensive thus its protocol is always revised by the standardization committees. Selenite cystine (SC) broth has been replaced

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by Rappaport Vassiliadis broth with soypeptone (RVS) within the ISO as well as the AOAC protocol (Van der Zee, 2003).

The International Dairy Federation (IDF) refused the controversely discussed ISO 6579:2000 draft and referred to its own method ISO 6785:2001 IDF 93. This standard procedure for the detection of *Salmonella* from milk and milk products is similar to the protocol EN 12824:1998 (Becker et al., 2003).

The aim of our study was to compare the draft of the horizontal method for the detection of *Salmonella* species from human food and animal feed (ISO 6579:2002) with the European gold standard (DIN EN 12824:1998) on the basis of raw porcine, bovine and poultry meat samples (spiked $n=92$; naturally $n=286$). Three new chromogenic plating media have been studied, because most of the earlier studies investigated clinical stool samples. Therefore, chromogenic media had a higher specificity but a lower sensitivity (Rambach, 1990; Dusch and Altwegg, 1993; Gaillot et al., 1999; Perez et al., 2003).

2. Materials and methods

2.1. Bacterial type strains

As summarized in Table 1 a total of 36 bacterial type strains were selected to assess the growth and appearance on six different plating media. This included non-typhoid *Salmonella* strains ($n=27$), different Enterobacteriaceae ($n=8$) and *Pseudomonas aeruginosa* ($n=1$).

All type strains were cultured in Brain Heart Infusion (BHI) broth (Merck KGaA, Darmstadt, Germany) under required conditions. The purity of the cultures was confirmed by streaking onto Plate count (PC) agar (Merck KGaA, Darmstadt, Germany). Subsequently the six different plating media: Brilliant Green Agar (BGA) according to Edel and Kampelmacher (Oxoid, Wesel, Germany); Xylose Lysine Deoxycholate (XLD) Agar (Sifin, Berlin, Germany); Xylose Lysine Tergitol 4 (XLT4) Agar (Merck KGaA, Darmstadt, Germany).

Miller–Mallinson (MM) Agar (Miller and Mallinson, 2000.); AES *Salmonella* Agar Plate (ASAP) Agar (AES Laboratoire, Combourg, France); and Oxoid *Salmonella* Chromogen Media (OSCM) Agar (Oxoid, Wesel, Germany) were inoculated by using standard multiple loop techniques.

2.2. Artificial inoculation of raw pork ground meat

Artificial spiking experiments were conducted using 1–10 colony-forming units ($n=25$), 11–50 CFU ($n=41$) and 51–200 CFU ($n=26$) concentrations of *S.* Typhimurium 164/93 BgVV in 25 g initial weight of raw ground pork meat samples ($n=92$). Prior to an oculation, all samples were initially confirmed as *Salmonella*-negative by gold standard. Artificial inoculation of other types of meats was beyond the scope of this study.

2.3. Naturally contaminated raw meat samples

A total of 286 meat samples comprising porcine and bovine minced meat ($n=206$) and poultry meat samples ($n=80$) including marinated poultry meat and poultry edible offal were examined for naturally occuring *Salmonella* contamination. All meat samples used in this study were bought at various local German grocery stores during the one-year trial period. Cultural enrichment investigation was performed as described below.

Table 1
Stock cultures ($n=36$) used

No.	Serotype or species	Identification
1	*Salmonella arizonae*	AES[a] 8.1.
2	*Salmonella* Bovismorbificans	IFTN[b] H 217
3	*Salmonella* Derby	BgVV[c] 1454/61
4	*Salmonella* Dublin	x-O 162/98[d]
5	*Salmonella* Dublin	x-O 163/98[d]
6	*Salmonella* Enteritidis	ATCC[e]13076
7	*Salmonella* Enteritidis	BgVV 164/93
8	*Salmonella* Enteritidis	IFTN W 28/8
9	*Salmonella* Give	IFTN W 37/8
10	*Salmonella* Goldcoast	IFTN H 9
11	*Salmonella* Hadar	IFTN W 30/1
12	*Salmonella* Infantis	IFTN H 21
13	*Salmonella* I-Rauhform	IFTN H 59
14	*Salmonella* Livingstone	IFTN H 19
15	*Salmonella* London	IFTN H 74
16	*Salmonella* Manhattan	IFTN W 33/4
17	*Salmonella* Newport	IFTN W 30/11
18	*Salmonella* Ohio	IFTN H 103
19	*Salmonella* Oranienburg	ATCC 3592
20	*Salmonella* Panama	IFTN H 100
21	*Salmonella* Paratyphi B	IFTN W 30/11
22	*Salmonella* Paratyphi B	IFTN W 35/11
23	*Salmonella* Senftenberg	DSM[f]10062
24	*Salmonella* Thompson	IFTN SV 4/7
25	*Salmonella* Typhimurium O:5	IFTN H 273
26	*Salmonella* Typhimurium	BgVV 2260/93
27	*Salmonella* Virchow	BgVV 174
28	*Citrobacter freundii*	AES 1.2.
29	*Citrobacter freundii*	IFTN M 50b
30	*E. coli*	ATCC 25922
31	*Enterobacter aerogenes*	BgVV 1799/89
32	*Hafnia alvei*	NCTC[g]8105
33	*Proteus mirabilis*	NCTC 11938
34	*Proteus morganii*	BgVV 696/84
35	*Shigella sonnei*	BgVV 7887/89
36	*Pseudomonas aeruginosa*	ATCC 15442

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[e] American Type Culture Collection (ATCC), Virginia, USA.
[f] Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Germany.
[g] National Collection of Type Cultures (NCTC), London, UK.

2.4. Preparation of culture media

Buffered peptone water (BPW) enrichment (Merck KGaA, Darmstadt, Germany), Selenite Cystine (SC) broth (Oxoid, Wesel, Germany), Rappaport Vassiliadis broth (RVS) with soypeptone (Merck KGaA, Darmstadt, Germany) and Tetrathionate broth (MKTTn) with Novobiocin according to Muller and Kauffman

(Biokontrol, France) were used. The three traditional plating media BGA, XLD and XLT4 and three new chromogenic plating media MM, ASAP and OSCM were prepared according to manufacturers' instructions. A cost implication was out of the scope of this study.

2.5. Cultural enrichment

A detailed description of the detection methods is given in Fig. 1. Briefly, the cultural enrichment protocol according to ISO 6579:2002 and DIN EN 12824:1998 included a two- step enrichment method, plating on two solid selective agars and a preliminary confirmation.

A serological (*Salmonella antisera* "Enteroclon Anti-*Salmonella* A-67", Sifin, Berlin, Germany) and a biochemical test (biochemical rapid test "api®20E", bioMérieux, France) provided preliminary confirmation of the species isolated. Final confirmation and characterization of the antimicrobial drug resistance pattern were performed by the German National Reference Laboratory for *Salmonella* species (NRL-Salm, Berlin, Germany). In this study a shortened incubation time of 24 h (according to ISO 6579:2002) as well as a prolonged cultivation time of 48 h (according to DIN EN 12824:1998) were performed for all enrichment steps. This means, that a *Salmonella* species positive result was obtained after six days according to ISO 6579:2002 or eight days according to DIN EN 12824:1998. A *Salmonella* species negative result was obtained after four or six days.

2.6. Statistical analysis

The statistical reliability of the results obtained for 286 naturally contaminated samples was proven by a non-parametric hypothesis. The null hypothesis defines:

The ISO 6579:2002 draft is no less effective than the gold standard.

The statistical testing of inferiority has been done with the McNemar sign test (McNemar, 1947).

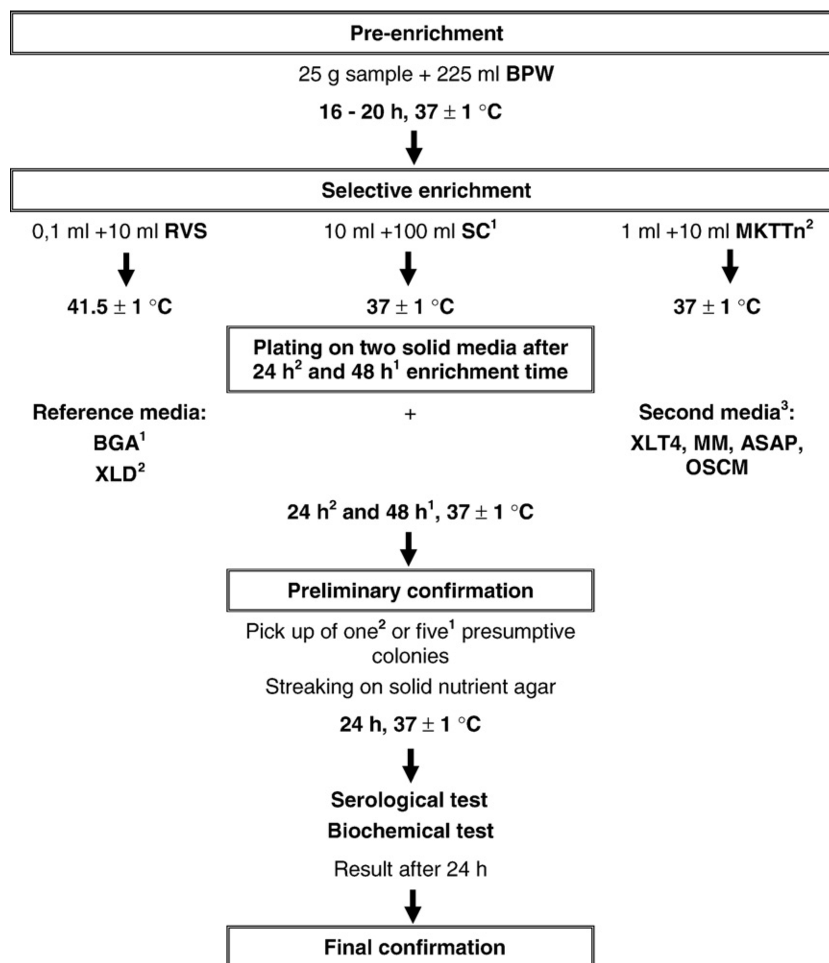

Fig. 1. Comparison of the horizontal method for the detection of *Salmonella* spp. (ISO 6579:2002) with the gold standard (DIN EN 12824:1998) regarding six different plating media.

3. Results

In this study the controversely discussed draft of the horizontal method for the detection of *Salmonella* species from human food and animal feed (ISO 6579:2002) was compared to the European gold standard (DIN EN 12824:1998) with regard to three new chromogenic plating media. For this purpose an assessment of six different plating media, an artificial inoculation of 92 raw ground pork meat samples and an investigation of 286 naturally contaminated raw porcine and bovine ground meat ($n=206$) and raw poultry meat samples ($n=80$) were performed.

3.1. Plating agar assessment

The growth and appearance of 36 stock isolates and type strains (*Salmonella* and other species) on the chromogenic agars MM, ASAP and OSCM were compared to those on the three traditional agar BGA, XLD and XLT4. A summary of the data is given in Table 2.

Twenty-six of the tested *Salmonella* type strains were strong in the production of hydrogen sulfide and negative lactose fermenters. *S.* Senftenberg DSM 10062 studied was an ultra-weak hydrogen sulfide-producing strain (Véron and Gasser, 1963; Miller and Mallinson, 2000). This strain was not detected by XLD and XLT4 agar. *Salmonella arizonae* AES 8.1 was a lactose-negative strain and did not show presumptive colonies on XLT4 and OSCM agar. *S.* Dublin x-O162/98 was not detected by using OSCM and ASAP agar. In addition, OSCM agar failed to detect *S.* Derby 1454/61, too. The two strains of *Citrobacter freundii* had *Salmonella*-like (H_2S-positive) colonies on BGA, XLD and XLT4 agar. The *Pseudomonas aeruginosa* strain gave false-positive results on BGA agar. MM agar detected all 27 stock *Salmonella* serotypes.

Furthermore, MM did not have any false-positives with any of the nine non-*Salmonella* stock cultures studied.

Table 2
Six different *Salmonella* plating media and detection problems for false-positive and false-negative stock *Salmonella* and non-*Salmonella* species

	Detection problems	
Plating media	False-positive	False-negative
BGA	*Citrobacter freundii*, *Pseudomonas aeruginosa*	None [a]
XLD	*Citrobacter freundii*	*Salmonella arizonae*, *S.* Senftenberg
XLT4	*Citrobacter freundii*	*Salmonella arizonae*, *S.* Senftenberg
MM	None	None
ASAP	None	*S.* Dublin
OSCM	None	*Salmonella arizonae, S.* Derby, *S.* Dublin

BGA — Brilliant Green Agar according to Edel and Kampelmacher
XLD — Xylose Lysine Deoxycholate Agar
XLT4 — Xylose Lysine Tergitol 4 Agar
MM — Miller–Mallinson Agar
ASAP — AES *Salmonella* Agar Plate Agar
OSCM — Oxoid *Salmonella* Chromogen Media Agar
[a] No failures observed.

Table 3
Percentage of *Salmonella* recoveries (%) from artificially spiked pork ground meat samples (n=41): 11–50 CFU S. Typhimurium/25 g pork ground meat

Incubation time	Culture media					
	BGA	XLD	XLT4	MM	ASAP	OSCM
				SC		
24 h[a]/24 h[b]	12,0	14,6	24,4	29,3	22,0	24,4
24 h/48 h	12,0	14,6	26,8	46,3	29,3	29,3
48 h/24 h	17,0	19,5	29,3	48,8	34,2	39,0
48 h/48 h	51,0	22,0	39,0	51,2	34,2	43,9
				MKTTn		
24 h/24 h	58,5	95,0	82,9	92,7	87,8	100
24 h/48 h	61,0	95,0	92,7	95,1	92,7	100
48 h/24 h	70,7	97,6	92,7	95,1	92,7	100
48 h/48 h	70,7	97,6	92,7	95,1	92,7	100
				RVS		
24 h/24 h	100	100	100	100	100	100

BGA — Brilliant Green Agar according to Edel and Kampelmacher
XLD — Xylose Lysine Deoxycholate Agar
XLT4 — Xylose Lysine Tergitol 4 Agar
MM — Miller–Mallinson Agar
ASAP — AES *Salmonella* Agar Plate Agar
OSCM — Oxoid *Salmonella* Chromogen Media Agar
SC — Selenite Cystine broth
MKTTn — Tetrathionate broth with novobiocin according to Muller and Kauffman
RVS — Rappaport Vassiliadis broth with soypeptone
[a] Incubation time for selective enrichment broth.
[b] Incubation time for solid plating media.

3.2. Artificially inoculated raw meat samples

A total of 92 ground pork samples were spiked with 1–10 CFU ($n=25$), 11–50 CFU ($n=41$) or 51–200 CFU ($n=26$) of *S.* Typhimurium in 25 g what is presented in Table 3. A shortened 24-hour-enrichment in RVS broth yielded a recovery rate of *Salmonella* of 100% from artificially spiked ground pork samples regardless of the plating media or inoculation level used. However, the traditional reference plating media BGA had a *Salmonella* recovery of 40% (1–10 CFU), 51% (11–50 CFU) and 61,5% (51–200 CFU) in combination with a prolonged 48-hour-enrichment in SC broth. Only a combination with the new chromogenic plating media MM significantly increased the *Salmonella* recovery rate of SC broth (60%, 51,2% and 84,6%). A shortened 24 h enrichment in MKTTn broth in combination with the new reference plating media XLD yielded a higher *Salmonella* recovery rate (68%, 95% and 96%). Even a shortened 24 h enrichment in MKTTn broth in combination with the new chromogenic plating media OSCM increased the *Salmonella* recovery rate up to 100% (80%, 100% and 100%).

3.3. Naturally contaminated samples

Considering all isolations made, a total of 39 (13,6%) of the 286 samples selected for investigation of naturally occuring *Salmonella* contamination were found to be *Salmonella*-positive (Table 4). With various broths and agars tested and considering a prolonged 48 h enrichment time, RVS broth

Table 4
Comparison of the horizontal method (ISO 6579:2002) with the gold standard (DIN EN 12824:1998) by using the McNemar sign test (McNemar, 1947)

		ISO Draft		Σ
		+	−	
Gold standard	+	35	1	36
	−	1	249	250
	Σ*	36	250	286
				$p=1{,}000$

*Outside these protocols, 2 *Salmonella*-positive samples have been detected after incubating in MKTTn broth and plating on OSCM or ASAP agar.

(97,4%) yielded a higher percentage of *Salmonella*-positive samples than MKTTn (94,9%) or SC (38,5%), respectively. The chromogenic plating media provided the best results regarding a shortened incubation time of 24 h in RVS in contrast to BGA, XLD and XLT4 (Table 5). For these samples, no statistical significant difference was obtained by comparing the ISO 6579:2002 draft to the gold standard. Therefore, the null hypothesis could not be refused. This means that the ISO draft is not inferior to the conventional method (Table 4).

3.4. Subtyping of serotypes, subtypes recovered and drug resistance patterns

In our investigations 43 different strains have been isolated from 39 naturally *Salmonella*-positive minced meat and fresh poultry samples. The prevalence of serotypes isolated was: *S.* Typhimurium ($n=21$) with the subtypes DT 104 L, DT 012 and RDNC, *S.* Infantis ($n=8$), *S.*

S. Saint-Paul ($n=3$), *S.* Senftenberg ($n=1$), *S.* Derby ($n=1$), *S.* I-Rauhform ($n=1$), *S.* Paratyphi B D-tartrate-positive ($n=1$), *S.* Enteritidis PT1 ($n=1$) and untypable strains from *Salmonella* group D1 ($n=5$) and B ($n=1$).

Strains from *S.* Paratyphi B D-tartrate-positive ($n=2$) and *S.* Saint-Paul ($n=3$) cultured from poultry meat, were multi-resistant to four or six antibiotics including nalidixic acid. Strains of *S.* Typhimurium DT 012 ($n=1$) and *S.* Typhimurium RDNC ($n=2$) isolated from bovine and porcine minced meat were also multi-resistant to eight antibiotics.

4. Discussion

4.1. Plating agar assessment

This study confirms that traditional plating media such as XLD (Garrick and Smith, 1994; Warburton et al., 1994) as well as chromogenic plating media like ASAP (Perez et al., 2003; Gray et al., 2003) and OSCM (Zewde, 2001) are not able to reliably detect some non-typhoid *Salmonella* strains. Furthermore, our findings support the observation that XLD as well as BGA and XLT4 often have false-positive colonial morphology for some strains of *Citrobacter freundii* (Warburton et al., 1994) and *Pseudomonas aeruginosa*. We have found that MM agar detected all 27 selected non-typhoid *Salmonella*, nine non-*Salmonella* type strains especially ultra-weak H_2S-producing and lactose fermenting *Salmonella* spp. (Miller and Mallinson, 2000).

4.2. Artificially inoculated raw meat samples

In this study an artificial inoculation of raw pork ground meat samples ($n=92$) revealed essentially identical results, especially for a RVS broth enrichment. This observation has been also noticed in previous studies (Edel and Kampelmacher, 1969; Van Leusden et al., 1982). Nevertheless, spiking experiments are essential for the evaluation of standard methods. In our artificial inoculation experiments, RVS and MKTTn broth seemed to be the most suitable selective enrichments for the detection of *S.* Typhimurium from meat samples with high and low levels of contamination. A combined enrichment of RVS and MKTTn broth for the detection of *Salmonella* spp. from miscellaneous food samples was also recommended by others (Hammack et al., 1999; Hammack et al., 2001).

4.3. Naturally contaminated samples

In this study no significant difference was obtained by comparing the ISO 6579:2002 draft to the gold standard. In a recent collaborative study of 21 laboratories, Feldsine et al. (2003) compared the ISO 6579 *Salmonella* culture procedure to the AOAC official method with an investigation of fresh cheese, poultry products and dried egg products. Feldsine et al. (2003) reported results compatible with our study. Furthermore, our study confirms that RVS broth is the most suitable enrichment for the investigation of raw meat, including marinated poultry meat and poultry edible offal (Arroyo and Arroyo, 1995). Thus, a combination of RVS broth and MKTTn broth appears acceptable. SC broth is less advisable for the isolating of

Table 5
Percentage of *Salmonella*-positive samples (%) detected from 286 naturally contaminated meat samples: initial recoveries after 24 h and total recoveries after 48 h incubation

Incubation time[a]	Culture media					
	BGA	XLD	XLT4	MM	ASAP	OSCM
				SC		
24 h	23,1	18,0	23,1	30,8	20,5	18,0
48 h	30,8	23,1	38,5	35,9	30,8	30,8
				MKTTn		
24 h	59,0	79,5	89,8	89,8	92,3	84,6
48 h	71,8	84,6	94,9	92,3	92,3	87,2
				RVS		
24 h	82,1	89,7	89,7	94,9	97,4	92,3
48 h	89,7	94,9	97,4	97,4	97,4	94,9

BGA — Brilliant Green Agar according to Edel and Kampelmacher
XLD — Xylose Lysine Deoxycholate Agar
XLT4 — Xylose Lysine Tergitol 4 Agar
MM — Miller–Mallinson Agar
ASAP — AES *Salmonella* Agar Plate Agar
OSCM — Oxoid *Salmonella* Chromogen Media Agar
SC — Selenite Cystine broth
MKTTn — Tetrathionate broth with novobiocin according to Muller and Kauffman
RVS — Rappaport Vassiliadis broth with soypeptone
[a] Incubation time for selective enrichment broth.

foodborne *Salmonella* spp. from raw meat and flesh (June et al., 1995). Similar to a recent study (Miller and Mallinson, 2000), we found that the chromogenic plating media MM, ASAP and OSCM gave the best results in contrast to BGA, XLD and XLT4.

4.4. Strain typing and drug resistance pattern

In this study, a representative spectrum of common serotypes and antimicrobial drug resistance, were found much as they are in other European countries. Multi-resistant *Salmonella* serotypes isolated from raw whole chicken has also been described in a recent British study (Jørgensen et al., 2002). *S.* Typhimurium DT 104 remains one of the most commonly found multi-resistant strains in the United Kingdom (Mansfield and Forsythe, 2000).

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