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Full Length Research Paper

Identification and antimicrobial resistance profiles of Salmonellae isolated from the broiler dressing plants associated with their environments

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The study was aimed at isolation and identification of *Salmonella* spp. from dressing water, device and environmental samples collected from pluck shops (cottage poultry processors) by using cultural characteristics, differential staining, biochemical techniques and motility test. Furthermore, the isolated *Salmonella* spp. was characterized by antimicrobial susceptibility testing. Among the 27 *Salmonella* isolates, 22.22% (n=6) were identified as *Salmonella pullorum* (SP), 18.51% (n=5) as *Salmonella gallinarum* (SG), 59.26% (n=16) as *Salmonella typhimurium* (ST). Motility test indicated that 40.74% (n=11) isolates were non motile and 59.26% (n=16) were motile. As regards source of isolates, 33.33% (n=2) of SP was equally available in dressing water, device and environmental samples. On the other hand, 40% (n=2) of SG was detected in dressing water, 20% (n=1) in device and 40% (n=2) in environmental samples. However, 50% (n=8), 31.25% (n=5) and 18.75% (n=3) ST was detected respectively in dressing water, device and environmental samples. Antibiogram studies indicated that SP, SG and ST were more or less susceptible to chloramphenicol (C), azithromycin (AZ), ciprofloxaxin (CIP) and norfloxacin (NOR) but SP was also susceptible to gentamycin (CN). Besides being resistant to a number of antibiotics, the organisms revealed also multidrug sensitivity that calls attention of practitioner on the use of antibiotics.

Key words: Broiler dressing plant, salmonellae, identification, antimicrobial susceptibility.

INTRODUCTION

Poultry industry is a rising profitable sector in Bangladesh. There has been tremendous development of this sector over the recent years in this county (Rahman 2003) which contributed significantly to economic development leading to almost an industry. According to the Bangladesh Bureau of Statistics (BBS) about 89% of the rural households' rear poultry. This rural farming is also emerging as a strong agro-based industry that includes the backyard poultry rearing system and commercial intensive one. However, the advancement of poultry industry is being seriously hampered due to outbreak of various infectious and noninfectious diseases. Among the bacterial infections diseases, Salmonellosis due to the organism belonging to the genus *Salmonella.* Salmonellae are Gram negative, short plump shaped rods, non-spore forming, non-capsulated, aerobic and facultatively anaerobic organisms belonging to the family Enterobacteriaceae (OIE, 2006).

Chicken meat is a primary source of *Salmonella* in which about 10% transmission of *Salmonella* occur through poultry meat (Gast, 1997). Isolated species from chicken meat was *S. gallinarum*, *S. pullorum*, *S. typhimurium*, *S. enteritidis*, *S. hiduddify* (Raufu *et al.*, 2009; Kim *et al.*, 2007; Roy *et al.*, 2002). Human stool acts as an important reservoir of *Salmonella* spp. Species isolated from human stool are *Salmonella typhi*, *S. paratyphi* A, *S. typhimurium*, *S. wrothington*, *S. enteritidis* (Kumar *et al.*, 2009; Kornschober *et al.*, 2009; Kariuki *et al.*, 2002).

Poultry birds have frequently been incriminated as a means of *Salmonella* contamination and consequently acting as a major source of the pathogen in humans. This organism has been isolated from a range of foods in almost every country (Rumeu *et al*., 1997). The level of contamination dramatically increases during the contain-

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ment of the animals(birds) in holding pens before slaughter and besides, the increasing incidence of salmonellosis was due to a number of technical practices (D'Aoust, 1994). After slaughter, the subsequent dressing of meat increases the spread of *Salmonella* on meat surfaces, and by the time the meat is in retail outlets, contamination levels might be increased by 20 % (Forsythe and Hayes, 1998). Most *Salmonella* infections in humans result from the ingestion of contaminated poultry, beef, pork, eggs, and milk (Gomez *et al.,* 1997). Along with increase of public health problem due to this pathogen (salmonellae), use of antimicrobials in any environment creates selection pressures that favor the survival of antibiotic-resistant pathogens. The routine practice of giving antimicrobials to domestic livestock including poultry for growth promotion and prophylaxis is an important factor in the emergence of antibiotic-resistant bacteria in the food chain (Tollefson *et al.,* 1997). Such a condition leads to investigation on the antibiogram nature and their resistance and sensitivity pattern to various antibiotics. In consideration of the above rationale in view, the present piece of research was undertaken with a view to isolate, identify and characterize Salmonellae from the broiler dressing plants associated with their environments.

MATERIALS AND METHODS

Study area

The source of samples included (dressing water, device and environmental swab) which were collected from Pluck shops (cottage poultry processors) located in and around BAU campus area (Kamal and Ranjit Market, and "*sash mur*") and then transported to the Bacteriological laboratory of the Department of Microbiology and Hygiene, BAU, Mymensingh for isolation, identification, and antimicrobial susceptibility testing.

Collection and transportation of samples

A total of 60 samples comprising of dressing water, devices and environmental swabs were collected and inoculated immediately into selenite broth for better nourishment of the desirable organisms and immediately brought to Bacteriology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh.

Cultural characterization and isolation of *Salmonella* spp.

The longitudinal surface of each samples was seared (cauterized) with hot spatula and incised with sterile scalpel followed by introduction of a loop in the cut surface and materials brought with loop was inoculated into Selenite broth and SS agar plates. These were then incubated at 37°C for 24 h in bacteriological incubator. After 24 h the incubated media were then examined for growth of bacteria. Colorless or translucent colony and sometimes black color colony were observed on SS agar. The colony was then subjected to Gram's Method of staining and observed under microscope for Gram negative rods. The organisms from the agar media were sub-cultured into SSA, MCA and BGA with the help of inoculating loop in case of gram negative rods in the smears. In case of SS agar colorless, translucent and black colony were observed. In case of MC agar colorless and translucent colony were observed. In case of BG agar, light pink colony against a rose pink background was observed. Thus single pure colony was obtained. These pure isolates obtaining in this way were used for the further study (Cheesebrough, 1985). The Salmonellae colonies were characterized morphologically using Gram's stain according to the method described by Merchant and Packer (1967). The motility test was performed to differentiate motile bacteria from non-motile one (Cheesbrough, 1985).

Differentiation of isolated *Salmonella* using biochemical test

For this study, isolated organisms with supporting growth characteristics of *Salmonella* were subjected to sugar (Carbohydrate) fermentation test, TSI agar slant reaction, MR-VP reaction, indole reaction, urease reaction, citrate utilization and Lysine decarboxylation reaction according to the procedures as described by Cheesbrough (1985).

Comparative antimicrobial sensitivity pattern of *Salmonella typhimurium, Salmonella gallinarum* and *Salmonella pullorum*

Susceptibility and resistance of different antibiotics was measured in vitro by employing the Kirby-Bauer (Bauer *et al*., 1996) method. This method allowed for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the disc.

A suspension of test organism was prepared in NB by overnight culture for 24 hours at 37^0C. The broth were streaked using sterile glass spreader homogenously on the medium. Antibiotic disc were applied aseptically on to the surface of the inoculated plates at an appropriate special arrangement with the help of a sterile pair of forceps on Mueller-Hinton agar plates. The plates were then inverted and incubated at 37^0C for 24 hours. The di-

Table.1 Interpretive standards for disc diffusion susceptibility testing.

Name of antibiotic Disc	Disc concentration	Diameter of zone of Inhibition (mm)		
		Sensitive	Intermediate	Resistant
Ampicillin (AMP)	10 µg	≥ 17	14-16	≤ 13
Chloramphenicol (C)	30 µg	≥ 18	13-17	≤ 12
Tetracycline (TE)	30 µg	≥ 15	12-14	≤ 11
Erythromycin (E)	15 µg	≥ 23	14-22	≤ 13
Azithromycin (AZ)	15 µg	≥ 18	14-17	≤ 13
Streptomycin (S)	10 µg	≥ 15	12-14	≤ 11
Gentamicin (CN)	10 µg	≥ 15	13-14	≤ 12
Nalidixic acid (NA)	30 µg	≥ 19	14-18	≤ 13
Ciprofloxacin (CIP)	5 µg	≥ 21	16-20	≤ 15
Norfloxacin (NOR)	10 µg	≥ 17	13-16	≤ 12

ffusion discs with antimicrobial drugs were placed on the plates and incubated for 24 hours at 37^0C. The antibiotics discs (Oxoid, Basingstoke, Hampshire, England) used were: Ampicillin(AMP), Chloramphenicol (C), Tetracycline (TE), Erythromycin (E), Azithromycin (AZ)), Streptomycin(S), Gentamicin (CN), Nalidixic acid (NA), Ciprofloxacin (CIP), Norfloxacin (NOR).

Sterile glass spreader was used to spread the culture homogenously on the medium. Antibiotic disc were applied aseptically to the surface of the inoculated plates at an appropriate special arrangement with the help of a sterile pair of forceps. The plates were then inverted and incubated at 37 ^{0}C for 24 hours. After incubation, the plates were examined and the diameters of the zone of complete inhibition were observed. Isolates were classified as susceptible, intermediate and resistant categories based on the standard interpretation table (Table 1) updated according to the Clinical and Laboratory Standards Institution (CLSI, 2011).

Maintenance of stock culture

Preservation of *Salmonella* isolates in pure culture form was stored in sterile 50% glycerin (50 parts pure sterile glycerin with 50 parts PBS) and was used as stock culture. The equal volume of 50 % glycerin and bacterial culture were mixed and capped tightly and stored at -80^0C. The isolated organisms were given code name for convenience.

RESULTS

A total of 27 bacterial isolates out of 60 samples were identified as *Salmonella* spp. by using cultural and biochemical techniques. The results of cultural, morphological and motility characteristics of the isolates of *Salmonella* spp. are presented in Table 2.

The results of percentages (%) of *Salmonella* spp. available in source samples were presented in Table 3. Out of 20 water samples 12 (60%) were positive for *Salmonella*. Out of 20 environmental samples 7 (35%) were positive for *Salmonella*. Out of 20 device swab samples 8 (40%) were positive for *Salmonella*. *Salmonella pullorum* was detected as 2 (33.33%) in water, 2 (33.33%) in device samples and 2 (33.33%) in environmental samples. Furthermore, *Salmonella gallinarum* was detected as 2 (40%) in water, 1 (20%) in device swab and 2 (40%) in environmental samples. *Salmonella typhimurium* was detected as 8 (50%) in water samples, 5 (31.25%) in device samples and 3 (18.75%) in environmental samples.

The results of antimicrobial susceptibility testing by disk diffusion method for *Salmonella* species against 10 chosen antimicrobial agents are presented in Table 4. Among 6 isolates of *Salmonella pullorum* isolates, 6 (100%) were resistant to ampicillin, tetracycline, erythromycin, streptromycin and nalidixic acid was most common findings and followed by intermediately resistant to chloramphenicol 4 (66.67%), azithromycin 4 (66.67%), gentamycin 2 (33.33%) and, ciprofloxacin 2 (33.33%). On the other hand, 6 (100%) were susceptible to norfloxacin and followed by susceptible to azithromycin 2 (33.33%) and ciprofloxacin 4 (66.67%). Out of 5 *Salmonella gallinarum* isolates, 5 (100%) were resistant to ampicillin, tetracycline, erythromycin, streptromycin and nalidixic acid, and resistant to azithromycin 4 (80%), gentamycin 3 (60%) respectively followed by intermediately resistant to chloramphenicol 1 (20%), gentamycin 2 (40%) and ciprofloxacin 4 (80%). On the other hand, 5 (100%) were susceptible to norfloxacin and followed by susceptible to

Table 2. Results of cultural, morphological and motility characteristics of the isolates of *Salmonella* spp. at a glance.

Colony morphology					Staining characteristics	Motility (Hanging drop method)
SS agar	**EMB agar**	**NA agar**	**TSI agar**	**BA**		
Translucent, black smooth, small round colonies	Pink Color, Circular, and Smooth colonies	Translucent, opaque, smooth colonies	Black color colonies against a yellow background	Non hemolytic and grey colonies	Pink short rod, gram negative bacteria arranged in single or paired	+Ve (*S. typhimurium*) -Ve *(S. pullorum, S. gallinarum)*

N.B: SS= Salmonella-Shigella , EMB = Eosine Methylene Blue , NA = Nutrient Agar, TSI = Triple Sugar Iron, BA = Blood Agar.

Table 3. Results of percentage (%) of *Salmonella* spp. available in source samples.

Name of organism	Percentage (%) of *Salmonella* spp. available in source samples		
	Water samples	**Device samples**	**Environmental samples**
Salmonella pullorum (6)	2 (33.33)	2 (33.33)	2 (33.33)
Salmonella gallinarum (5)	2 (40)	1 (20)	2 (40)
Salmonella typhimurium (16)	8 (50)	5 (31.25)	3 (18.75)
Total = 27	12	8	7

chloramphenicol 4 (80 %), azithromycin 1 (20 %) and ciprofloxacin 1 (20). The isolates of *Salmonella typhimurium* isolates (16), 16 (100%) were resistant to ampicillin, tetracycline, erythromycin, streptromycin, nalidixic acid, and resistant to azithromycin 13 (81.25%), gentamycin 13 (81.25%) followed by intermediately resistant to chloramphenicol 8 (80%), gentamycin 3 (18.75%), ciprofloxacin 6 (37.5%). On the other hand, 5 (100%) were suscep-

Table. 4 Results of antimicrobial susceptibility of *Salmonella* spp.

Name of isolates	**No. (%)**									
Salmonella pullorum (n=6)	AMP	C	TE	E	AZ	S	CN	NA	CIP	NOR
Susceptible	0 (0)	2 (33.33)	0 (0)	0 (0)	2 (33.33)	0 (0)	4 (66.67)	0 (0)	4 (66.67)	6 (100)
Intermediate	0 (0)	4 (66.67)	0 (0)	0 (0)	4 (66.67)	0 (0)	2 (33.33)	0 (0)	2 (33.33)	0 (0)
Resistant	6 (100)	0 (0)	6 (100)	6 (100)	0 (0)	6 (100)	0 (0)	6 (100)	0 (0)	0 (0)
Salmonella gallinarum (n=5)										
Susceptible	0 (0)	4 (80)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	1 (20)	5 (100)
Intermediate	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	2 (40)	0 (0)	4 (80)	0 (0)
Resistant	5 (100)	0 (0)	5 (100)	5 (100)	4 (80)	5 (100)	3 (60)	5 (100)	0 (0)	0 (0)
Salmonella typhimurium (n=16)										
Susceptible	0 (0)	8 (50)	0 (0)	0 (0)	3 (18.75)	0 (0)	0 (0)	0 (0)	10 (62.5)	16 (100)
Intermediate	0 (0)	8 (50)	0 (0)	0 (0)	0 (0)	0 (0)	3 (18.75)	0 (0)	6 (37.5)	0 (0)
Resistant	16 (100)	0 (100)	16 (100)	16 (100)	13 (81.25)	16 (100)	13 (81.25)	16 (100)	0 (0)	0 (0)

Ampicillin (AMP), Chloramphenicol (C), Tetracycline (TE), Erythromycin (E), Azithromycin (AZ), Streptromycin (S), Gentamycin (CN), Nalidixic acid (NA), Ciprofloxacin (CIP), Norfloxacin (NOR).

tible to norfloxacin and followed by susceptible to chloramphenicol 8 (50 %), azithromycin 3 (18.75 %) and ciprofloxacin10 (62.5 %).

The results of antimicrobial resistance patterns of *Salmonella* species are summarized in Table 5. Out of 6 *Salmonella pullorum* isolates, 2 (33.33%) was resistant to 5 antimicrobial agents and 4 (66.66%) was resistant to each of 6 antimicrobial agents. Out of 5 *Salmonella gallinarum* isolates, 1 (20%) and 2 (40%) were resistant to 6 antimicrobial agents. On the other hand, 2 and 2 (40%) were resistant to 7 antimicrobial agents.

Out of 16 *Salmonella typhimurium* isolates, 1 (6.25%) were resistant to 5 antimicrobial agents. Furthermore, 1 (6.25%), and 2 (12.5%) were resistant to 6 antimicrobial agents respectively. Moreover, 1 (6.25%) and 4 (25%) were resistant to 7 antimicrobial agents and 7 (43.75%) were resistant to 8 antimicrobial agents. The results of frequency distribution of multidrug resistant isolates of *Salmonella* spp. are summarized in

Table .5 Results of Antimicrobial resistance pattern of *Salmonella* spp.

Isolates	Resistance profiles	No of isolates (%)
S. pullorum (n=6)	No resistance demonstrated	-
	a. Resistance to 5 agents (AMP-TE-E -S-NA)	4 (66.67)
	b. Resistance to 6 agents (AMP-TE-E-AZ-S-NA)	2 (33.33)
	Resistance isolates	n =6 (100%)
S. gallinarum (n=5)	No resistance demonstrated	-
	a. Resistance to 6 agents (AMP-TE-E –S-CN--NA)	1 (20)
	b. Resistance to 6 agents (AMP-TE-E- Az-S-NA)	2 (40)
	c. Resistance to 7 agents (AMP-TE-E -S-CN-NA)	2 (40)
	Resistance isolates	n = 5 (100%)
S. typhimurium (n=16)	No resistance demonstrated	-
	a. Resistance to 5 agents (AMP-TE-E-AZ- NA)	1 (6.25)
	b .Resistance to 6 agents (AMP-TE-E-AZ- S- NA)	1 (6.25)
	c. Resistance to 6 agents (AMP-TE-E-S- CN-NA)	2 (12.5)
	d. Resistance to 7 agents (AMP-C-TE-E-AZ-S- NA)	1 (6.25)
	e. Resistance to 7 agents (AMP-TE-E- AZ-S- CN-NA	4 (25)
	f. Resistance to 8 agents (AMP-C-TE-E-AZ-S- CN-NA)	7 (43.75)
	Resistance isolates	n = 16 (100 %)

Table.6 Frequency distribution of multidrug resistant isolates of dressing water, device and environmental swabs. (When considered resistant to 2 or more drugs).

Name of isolates	No. (%)
Salmonella pullorum	6 (100)
Salmonella gallinarum	5 (100)
Salmonella typhimurium	16 (100)

Table 6 on the basis of resistant to 2 or more drugs. 6 (100%) *Salmonella pullorum*, 5 (100%) *Salmonella gallinarum* and 16 (100%) *Salmonella typhimurium* were detected as multidrug resistant isolates.

DISCUSSION

This study was aimed at isolation, identification and anti-biogram studies of *Salmonella* spp. recovered from broi-lers dressing plants and associated with their environ-ments of Pluck shops (cottage poultry processors) located in and around BAU campus area. During the isolation, identification of bacterial colonies having typical cultural characteristics was selected as presumptive for *Salmonella* serovers. For this, general purpose and differential selective media such as BA, NA, SSA, TSIA and EMB were used to culture the organism although all of them are not found equally suitable for all the serovars of *Salmonella*. In the present study, specific enriched media and biochemical tests mentioned above were also used by a number of researchers (Amin, 1969; Buxton and Fraser, 1977; Cheesbrough, 1985; Hossain, 2002; Habrun and Mitak, 2003; and Lee *et al*., 2003). The colony characteristics of *Salmonella* spp. such as translucent, black or colorless, smooth, small round colonies on SS agar; translucent, opaque, smooth colonies on Nutrient agar (NA) and black colored colonies on TSI agar were similar to the findings of other authors (Amin, 1969; Buxton and Fraser, 1977; Sujatha *et al*., 2003; Hossain 2002; Muktaruzzaman *et al*., 2010).

In Gram's staining, the morphology of the isolated sal-

monellae exhibited Gram negative characteristics of small rod shaped, single or paired in arrangement under microscope as was reported by other researchers (Cheesbrough, 1985; Freeman, 1985). Most of the source water of pluck shopkeepers' was Tube-well water while the other sources were water stored in tank as collected from local water pump. No *Salmonella* spp. were detected in former source like tube well water but very minute load of *Salmonella* spp. was detected from the latter stored tanks water. In case of motility test, performed by hanging drop slide method, 59.25% (where n=16) isolates were motile and 40.74% isolates (where n=11) were non motile. Motility test was fundamental basis for the detection of motility or otherwise characteristics of *Salmonella* organisms (Hossain, 2002; Freeman, 1985; and Buxton and Fraser, 1977).

In carbohydrate fermentation test, the isolates (n=27) that fermented glucose, maltose and produced acid and gas but did not ferment lactose those indicated positive for *Salmonellae* as was stated by Buxton and Fraser (1977). Among the 27 positive *Salmonella* isolates, 22.22% (where n=6) fermented glucose, maltose, rhamnose and produced both acid and gas but did not ferment dulcitol was considered positive for *S. pullorumum*. Only 18.51% isolates (where n = 5) fermented glucose, maltose, dulcitol without producing acid and gas and did not ferment rhamnose indicating typical characteristics of *S. gallinarum*. The rest 59.26% isolates (where n=16) fermented glucose, maltose, rhamnose and dulcitol with or without gas demonstrated provided indication of being *S. typhimurium*. These observations are strongly correlated with the theme of Lee *et al.,* (2003), Sujatha *et al*., (2003); Kwon *et al.,* (2010). A total of 27 (where n=27) isolates were positive for Methyl Red test but negative for VP test indicating characteristics of *Salmonella* spp. test which was similar with the statement of Muktaruzzaman *et al.,* (2010). In indole test, all the test isolates (where n=27) did not develop any red color that indicated the *Salmonella* isolates were negative to indole test (Lee *et al.,* 2003). Organisms isolated from the collected samples under test, revealed unequivocal morphological, cultural and biochemical properties resembling *Salmonella* spp as was recorded by Amin, (1969); Buxton and Fraser, (1977); Freeman (1985), and Hossain (2002).

Among the 27 isolates (where n=27) of 6 isolates were similar to *Salmonella pullorum* and only 5 isolates showed the characteristics of *Salmonella gallinarum* and 16 isolates were more or less similar to *Salmonella typhimurium.* The study also indicated that the field sample contained Gram negative, rod shape and motile organism with various colony characteristics (large, smooth, round and sticky) in different bacteriological media. The isolates was able to produce characteristic black metallic sheen colonies on EMB agar, pink colony on, pinkish colony on SS agar, circular, raised, smooth, colorless colony on NA.

Antimicrobial test was performed by disc diffusion method using 10 different commonly used antimicrobial agents. The isolates of *Salmonella pullorum* (6) were susceptible to norflaxin (100%), ciprofloxacin (66.67%) and gentamycin (66.67%) while 100% were resistant to ampicillin, tetracyclin, erythromycin, streptomycin, and nalidixic acid, followed by 66.67% intermediately resistant to chloramphenicol and azithromycin. On the other hand, the isolates of *Salmonella gallinarum* (5) were susceptible to norflaxin (100%) and 80% to chloramphenicol while resistant 100% to ampicillin, tetracycline, erythromycin , streptomycin, and nalidixic acid and 80% resistant to azithromycin, 60% to gentamycin followed by intermediately resistant to chloramphenicol (20%), to gentamycin (40%) and ciprofloxacin (80%). As regards the isolates of *Salmonella typhimurium* (16) these were susceptible to norflaxin (100%), ciprofloxacin (62.5%), chloramphenicol (50%), azithromycin (18.75%) while 100% resistant to ampicillin, tetracyclin, erythromycin, streptomycin, nalidixic acid and (81.25%) resistant to azithromycin and gentamycin followed by intermediately resistant to chloramphenicol (50%), azithromycin (18.75%) and ciprofloxacin (37.5%). Out of 27 *Salmonella* isolates, *Salmonella typhimurium* 16 (100%), *Salmonella gallinarum* 5 (100%) and *Salmonella pullorum* 6 (100%) were detected as multidrug resistant. Manie *et al*. 1998, also found several strains of multiple antimicrobial resistant *Salmonella* spp. in chicken. Recently, some authors have reported an increase in quinolone (Enrofloxacin) resistance in salmonella (Molbak *et al.,* 2002; Kabir, 2010; Tuhin-Al-Ferdous *et al.,* 2013) which also partially supports the findings of this study.

Further studies calling for attention for future research might be molecular characterization and genomic studies to have an idea about genes responsible for pathogenecity and drug resistance of the isolates of Salmonellae from washing water, device and environmental swab from broilers dressing plants as well their environments.

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