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Comparative analysis of multi-drug resistance pattern of *Salmonella sp.* isolated from chicken feces and poultry meat in Dhaka city of Bangladesh

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Abstract: *The study was conducted for comparative analysis of multi-drug resistance pattern of Salmonella sp isolated from chicken feces and poultry meat and characterization of the isolated using biochemical, and antimicrobial sensitivity techniques. A total of 20 samples were collected of which 50% were positive to Salmonella. All the culturally positive isolates fermented dextrose, maltose and mannitol with the production of acid and gas but not fermented sucrose and lactose. The same isolates showe Indole and V-P tests negative but M-R test positive. All the culturally and biochemically positive Salmonella. The antimicrobial susceptibility testing showed that the isolated Salmonella were highly sensitive to Ciprofloxacin and moderately sensitive to Gentamycin, kanamycin, Erythromycin and Nalidixic acid. However, the positive isolates were resistant to Azithromycin. The present study indicates that ciprofloxacin can be used as a first line therapy for the treatment of Salmonella gastroenteritis.*
Keywords: *MDR, Salmonella, Salmonellosis, Gastroenteritis, Poultry chicken, feces*

I. Introduction:

Salmonella causes various infections in humans. Contamination of people by *Salmonella* may be caused by infected persons, animals and direct contact of those with fluids *Salmonella* also has an important role in producing pathogens that cause food poisoning. *Salmonellas* act as primary reservoir for foods such as chicken meat, milk and milk products, eggs and meat products etc. Some of microorganisms such as Coliform bacteria have same features with *Salmonella*.

Salmonella species are responsible for an estimated 93.8 million cases of food borne disease in humans and an average of 155,000 deaths annually worldwide. Poultry and poultry meat product are considered one of the main carriers of the organism and represent a significant share of the attributed sources of salmonellosis in humans. The widespread occurrence of *Salmonella* in natural environment and the intensive husbandry practice used in the meat, fish and shellfish industries has been a significant problem in public health.Human Salmonella infection can lead to several clinical conditions including enteric fever, enterocolitis and systemic infections [49].Contamination of poultry carcasses with *Salmonella* seems to be mostly linked to flock contamination during rearing and/or transportation to slaughter. Risk factors for flock colonization by *Salmonella* include season, hatchery of origin, feed mills and various hygienic measures.

Surveillance of *Salmonella* in all the different stages of feed-food chain constitutes an important element in the exploration of epidemiology of food borne salmonellosis, and in the development and implementation of efficient *Salmonella* control strategies.

Salmonella is generally identified as being a non-lactose fermenting, (NLFs). Gram negative rod shaped organism, ranging 0.7 to 1.5 x 2 to 5 μm in size. *Salmonella* is oxidase negative, catalase positive, indole and Voges Proskauer (VP) negative, methyl red and Simmons citrate positive, H2S producing and urea negative. Some of these characteristics are used for biochemical confirmation of *Salmonella*. *Salmonella* infection in poultry generally causes no clinical symptoms, but nevertheless it can cause severe disease. Poultry can become infected with many different types of salmonella; about 50 percent of all *Salmonella spp*. have been detected in poultry.

In most cases, the birds are not sick and the production is not affected. The degree of illness depends on factors of both the bacteria and the host. The bacterium's serotype and phage type is of significance but also the type of animal, age and general health status.

Since antimicrobials started to be widely used by humans at the end of the 1940s, the emergence of resistant strains was observed in most bacterial species, and against all drugs available [50]. The irrational and widespread use of these agents has added to the problem, and resistance rates vary from place to place, depending on the local use of antibiotics.

II. Methods and materials:

Sample collections and processing:

A total of 34 poultry samples were collected Dhaka City. Sample included poultry feces and poultry meat .Feces samples were collected in clear, transparent ,sterile, wide mouthed bottles .Sample of poultry meat were collected by swabing the skin of slaughtered chicken whose feathers have been removed .Then the cotton swab was placed in a clean,sterile container. There were 17 meats and 17 poultry feces with different location. Among this 15 *Salmonella* found in poultry meat and 7 *Salmonella* found in feces. The highest number of *Salmonella* found in poultry meat.

3.2 Isolation and identification of *Salmonella*

3.2.1 Use of selective and differential media:

Samples were diluted in distilled water and then spread upon differential and selective media for *Salmonella* e.g. onto MacConkey agar, Xylose Lysine Deoxycholate agar (XLD) and also on Mannitol salt agar(MSA). Colony morphology (size, shape, margin, elevation, pigmentation etc.) were carefully observed after 18 h to 24 h (overnight) of incubation.

On the basis of colony morphology and characteristics on several differential and selective media, presumptively identified *Salmonella* colonies were subcultured into plates for further use. Pure cultures were undergone further microscopic and biochemical profiling.

3.2.2 Bacterial Count:

Plate count was restricted to 30-300 Colonies and plate containing more than 300 colonies were designed as too numerous to Count(TNTC) and plate Containing fewer than 30 Colonies were designed as too few count(TFTC).The following formula was uased for enumeration:

3.2.3 Microscopy

Microscopic studies such as cell size, shape, arrangements and Gram reaction were observed in a bright field microscope (Olympus, Japan) using magnification of 100× under oil immersion lens.

3.2.4 Identification of Micro-organism:

Idetification of bacterial isolate was carrid out by different bio-chemical test such as.Triple sugar Iron(TSI).Motality Indole Ureas(MIU).Methyl Red(MR).Voges proskaur(VP). Oxidase test.Catalase test.

3.2.4.1 Catalase

The test was done to differentiate bateria that produce the enzyme catalase producing ones. It was done by picking a pure colony by a steril loop and immersing it in 2-3 ml of the 3% H_2O solution in a glass slide production of bubbles indicated the positive results.

3.2.4.2 Oxidase

The test was done to detect the presence of cytochrome oxidase in the organism. A single colony was picked up with a sterile toothpickn and rubbed on to whatman filter paper that is soaked with 2-3 drops of dyes. Positive result was recognized by a dark purple color within 5-10 min.

3.2.4.3 Kleigler iron agar (KIA)

This test was performed to assess the mode of sugar utilization by stabbing the butt and streaking the slant of KIA media .after incubation at 37c for 18-24 h , result were recorded for change in color of butt &slant ,H_2S or other gas production.

3.2.4.4 Motility Indole Urea (MIU):

One Suspected isolated colony was touched with a sterile wire and stabbed into agar very carefully down the tube,without touching the bottom.The tube was incubated at 37c for 18 to 24 hours.

3.2.4.5 Methy red-Voges proskauer(MR-VP):

Isolated colonies were touched with sterile loop and MR-VP broth was inoculated by mean of loop inoculation.Tubes was inoculated at 37oC for 18-24 hours.

3.2.5 Determination of antibiotic Susceptibility of *Salmonella* isolates:

Susceptibility of *Salmonella* isolates to different antimicrobial agents was measured in vitro by the Kirby-Bauer method . It allowed rapid determination of the efficacy of drug by measuring the zone of inhibition that result from diffusion of the antimicrobial agent into the medium surrounding the disc. Commercially

available antimicrobial discs were used for the test.

Table 3.3 List of antimicrobial agents tested against the isolates:

Antimicrobial agent	Concentration (µg/mL)
Nalidixic Acid	20
Ciprofloxacin	5
Erythromycin	15
Amplicilin	10
Tetracycline	30
Gentamycin	10
Rifampicin	5
Streptomycin	10
Kanamycin	30
Azithromycin	15

III. Result:

A total of 20 poultry samples collected in Primeasia University laboratory during the study period were studied for the various bacterial pathogen. There were 10 meats and 10 poultry feces with different location. Among this 6 *Salmonella* found in poultry and 4 *Salmonella* found in feces. The highest number of *Salmonella* found in poultry meat.

Table 4.1: Growth of isolates on various agar plate

Sample No	Poultry Feaces			Poultry Meat		
	E.coli	***Staphylococcus aureus***	***Salmonella***	***E.coli***	***Staphylococcus aureus***	***Salmonella***
S-1	+	-	-	+	-	+
S-2	+	+	-	-	-	+
S-3	+	+	-	-	-	+
S-4	+	+	-	+	+	-
S-5	-	+	-	+	+	-
S-6	+	-	-	+	+	-
S-7	-	-	-	+	+	-
S-8	-	+	-	+	+	-
S-9	-	-	-	+	-	-
S-10	+	+	+	+	+	-
S-11	-	-	-	+	+	-
S-12	-	-	-	+	+	+
S-13	-	-	-	+	+	+
S-14	-	-	-	+	+	-
S-15	-	-	-	+	+	-
S-16	-	-	+	+	-	-
S-17	+	+	-	-	-	-
S-18	+	-	+	+	-	-
S-19	+	-	+	-	-	-
S-20	+	-	-	-	-	+

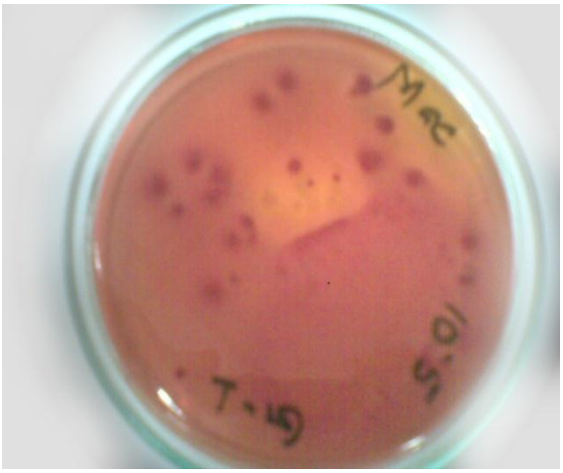


Fig 4.1: Growth on MacConkey agar plate

4.3 Total sample count:

After incubation of the plates,total coliform count,total *Staphylococcus* count, and total *Shigella Salmonella* count were carried out to measure CFU/gm of samples. Plate count was restricted to 30-300 Colonies and plate containing more than 300 colonies were designed as too numerous to Count (TNTC) and plate Containing fewer than 30 Colonies were designed as too few count(TFTC).

The following formula was used for enumeration.

Number of cell per ml= number of colonies (x) dilution factor /Volume of sample used(Cfu/g).

Sample	TCC cfu/g	TSSC cfu/g	TSC cfu/g
Sample-1	None	$18.8x10^6$	None
Sample-2	13.2×10^6	None	TNTC
Sample-3	None	None	9.5×10^5
Sample-4	10×10^6	1.2×10^4	None
Sample-5	None	9.2×10^5	None
Sample-6	6.8×10^6	None	None
Sample-9	None	None	10.0×10^5
Sample-10	9×10^6	6×10^6	None
Sample-12	None	6.5×10^5	None
Sample-13	6.7×10^5	None	None
Sample-16	$12.\times10^5$	7.2×10^5	None
Sample-17	13.2×10^5	8.8×10^5	9.8×10^5
Sample-18	21×10^5	None	18.8×10^5
Sample-19	None	None	10.8×10^5
Sample-20	None	9×10^5	None

Table 4.2: Total Microbial count in poultry meat and feces.

4.4 Total isolated pathogen:

After determining total sample count, pathogen was presumably identified from both poultry meat and feces sample 20 samples in total.

Table 4.3: Different isolated pathogen.

Total number of sample studied(20)	Different Isolated Pathogen					
20	Poultry feces			Poultry meats		
	Salmonella sp	*E.coli*	*S. aureus*	*Salmonella sp*	*E.coli*	*S. aureus*
	4(20%)	10(50%)	7(35%)	6(30%)	15(75%)	11(55%)

4.5 Bio-chemical test:

Biochemical test were done for the identification for isolates. Identification of bacterial isolate was carried out by different bio-chemical test such as. Triple sugar Iron (TSI).Motility Indole Urease(MIU).Methyl Red(MR).Voges proskaur(VP). Oxidase test. Catalase test. Sample from poultry meat and poultry feces were recorded separately for comparative analysis.The results of biochemical test are documented in tables:

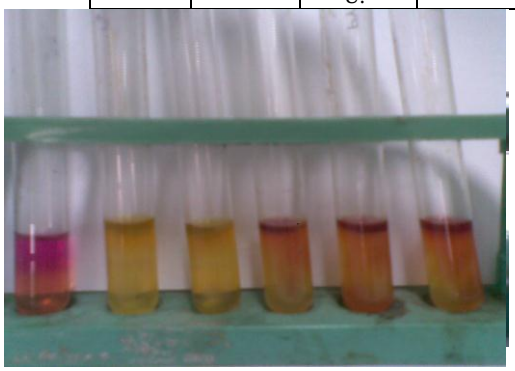
Table 4.4: Biochemical result of poultry meat.

Sample No	Gram Staining	MIU	KIA	MR	VP	Catalase test	Oxidase	Presumptive Organism
S-1	Pink Rod	M:- I:+ U:+	Butt-Red Slant-Red	+	-	+	-	*Salmonella spp*
S-2	Pink Rod	M:- I:- U:-	Butt=Red Slant=Red	+	-	+	-	*Salmonella spp*
S-3	Pink Rod	M:- I:-	But=Red Slant=Red	+	-	+	-	*Salmonella spp*

		U:-						
S-12	Pink Rod	M:- I:+ U:-	Butt=Black Slant=Red	+	-	+	-	*Salmonella spp*
S-13	Pink Rod	M:- I:- U:+	Butt=Black Slant-Red	+	-	+	-	*Salmonella spp*
S-20	Pink Rod	M:- I:- U:+	Butt=Black Slant=Red	+	-	+	-	*Salmonella spp*

Table 4.5: Biochemical result of poultry meat.

Sample No	Gram staining	MIU	KIA	MR	VP	Catalase	Oxidase	Presumptive Organism
S-10	Pink Rod	M:- I:+ U:-	Butt=Black Slant=Red	+	-	+	-	*Salmonella spp*
S-16	Pink Rod	M:- I:- U:-	Butt=Black Slant=Red	+	-	+	-	*Salmonella spp*
S-18	Pink Rod	M:- I:- U:-	Butt=Black Slant=Red	+	-	+	-	*Salmonella spp*
S-19	Pink Rod	M:- I:+ U:-	Butt=Black Slant=Red	+	-	+	-	*Salmonella spp*



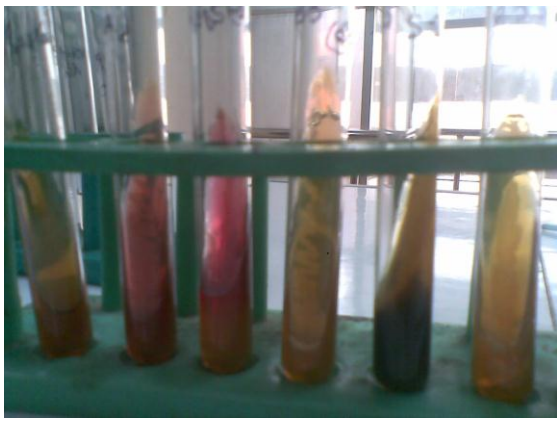


Fig 4.3: MIU test results. One on left is control.

Fig 4.4: MR-VP test result.

Fig 4.5: TSI agar slant.

4.6 Antibiotic Susceptibility Patters of Isolated *Salmonella*:

After 24 hour of incubation,to determine sensitivity inoculated Muller – Hinton agar plates were observed. Isolates from poultry products identified as *Salmonella* were introduced antibiotic susceptibility test by Kirby –Bauer method. Sensitivity pattern of *Salmonella* is as follows:-

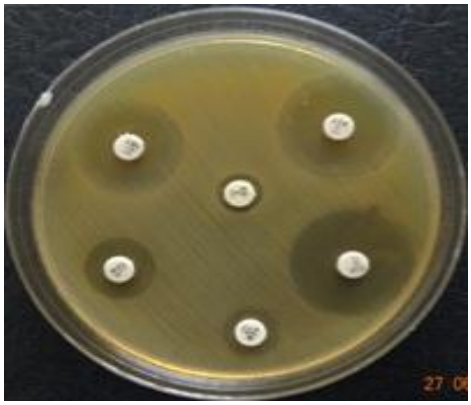


Fig 4.6: Determination of antibiotic sensitivity of isolates.

Table 4.6: Antibiotic sensitivity of isolates.

Sample type	Sample No	NAL 30□g	CIP 5□g	ERY1 5□g	TET 30□g	AMP 10□g	GEN 10□g	RIF 5□g	STP10□g	KAN 30□□g	AZM 15□□g
Poultry Meat	S-1	R	R	R	R	R	R	R	R	R	R
	S-2	S	S	I	R	S	I	R	S	I	R
	S-3	R	S	R	R	R	R	R	R	R	R
	S-12	I	S	R	R	R	R	R	R	S	R
	S-13	I	R	R	I	R	R	R	R	R	R
	S-20	R	I	R	R	S	R	R	R	I	R
Poultry Feces	S-10	R	I	R	R	I	I	R	R	I	R
	S-16	S	S	R	R	R	S	R	I	S	R
	S-18	R	R	R	R	R	I	R	R	R	R
	S-19	R	S	R	R	S	S	I	S	S	R

Table 4.7: Antibiotic Resistance of salmonella isolates.

Antibiotic disc	Resistance	Intermediate	Sensitivity
NA 30	60% (6/10)	20% (2/10)	20% (2/10)
CIP 5	30% (3/10)	20% (2/10)	50% (5/10)
ERY 15	90% (9/10)	10% (1/10)	-
TET 30	90% (9/10)	10% (1/10)	-
AMP10	60% (6/10)	10% (1/10)	30% (3/10)
GEN 10	50% (5/10)	30% (3/10)	20% (2/10)
RIF 5	90% (9/10)	10% (1/10)	-
STP 10	70% (7/10)	10% (1/10)	20% (2/10)
K 30	40% (4/10)	30% (3/10)	30% (3/10)
AZM15	100% (10/10)	-	-

Fig 4.7: Antibiotic resistance pattern against *Salmonella.*

IV. Discussion:

Poultry is growing and an under develop industry in Bangladesh. In recent years, many diesease of poultry animals have been seen in Bangladesh. Mostly, those diseases were caused by pathogen which was foreign or emerging pathogens. *Salmonella* and disease caused by *Salmonella* are seen often. However, the pathogen and disease caused by it are not abundant.

In-vitro antibiotic sensitivity pattern of isolated *Salmonellae* was performed against 10 commonly used antibiotics belonging to different groups. After incubation, plates were examined and diameters of the zone of inhibition for individual antibacterial agents were designated as highly sensitive, moderately sensitive, less sensitive and resistant (Table **4.6**).

Among the isolates 50% were highly sensitive to ciprofloxacin, 30% and were to Amplicilin and Kanamycin respectively while 20% to Nalidaxic acid and Streptomycin. However 10% were moderately sensitive to Erythromycin and Tetracyclin while were Streptomycin, Rifampicin. 30%, 20% and 10% were found to be less sensitive to Gentamycin, Nalidixic acid and Rifampicin. As regard effectivity of the antibiotics, 100% were highly resistant to Azithromycin and 90% resistant to Erythromycin while 70% were resistant to Streptomycin and 60% Nalidixic acid(Table:**4.7**).Rest of the antibiotics exhibited medium antimicrobial activity against the isolates.

The development and use of antibiotic has been one of the most important steps towards controlling of infectious bacterial diseases in 21st century. However, the subsequent appearance and spread of antibiotic

resistance in pathogenic organisms have made many currently available antibiotics ineffective. To successfully fight the increasing number of drug resistant and multi drug resistance bacteria, extensive knowledge of the molecular mechanisms of acquiring antibiotic resistance and updated information is required. From the present study, it could be concluded that public health awareness should be developed to reduce the incidence of *Salmonellosis* among the people in order to avoid food borne illness. Proper treatment should be done with strict sanitary measures

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[37]. Stokes, J.L.; W.W. Osborne, H.G. Bayne (1956 September). "Penetration and Growth of *Salmonella* in Shell Eggs". Journal of Food Science 21 (5): 510–518. doi:10.1111/j.1365-2621.1956.tb16950.x. "Normally, the oviduct of the hen is sterile and therefore the shell and internal contents of the egg are also free of microorganisms (10,16). In some instances, however, the ovaries and oviduct may be infected with Salmonella and these may be deposited inside the egg (12). More frequently, however, the egg becomes contaminated after it is laid."

[38]. Okamura, Masashi; Yuka Kamijima, Tadashi Miyamoto, Hiroyuki Tani, Kazumi Sasai, Eiichiroh Baba (2001). "Differences Among Six *Salmonella* Serovars in Abilities to Colonize Reproductive Organs and to Contaminate Egges in Laying Hens". Avian Diseases 45 (1): 61–69. doi:10.2307/1593012. JSTOR 1593012. PMID 11332500. "when hens were artificially infected to test for transmission rate to yolks: "Mature laying hens were inoculated intravenously with 106 colony-forming units of Salmonella enteritidis, *Salmonella typhimurium, Salmonella* infantis, *Salmonella* hadar, *Salmonella* heidelberg, or *Salmonella* montevideo to cause the systemic infection. Salmonella enteritidis was recovered from three yolks of the laid eggs (7.0%), suggesting egg contamination from the transovarian transmission of S. enteritidis.""

[39]. Gast, RK; D.R. Jones, K.E. Anderson, R. Guraya, J. Guard, P.S. Holt (August 2010). "In vitro penetration of *Salmonella Enteritidis* through yolk membranes of eggs from 6 genetically distinct commercial lines of laying hens". Poultry Science 89 (8): 1732–1736. doi:10.3382/ps.2009-00440. PMID 20634530. Retrieved 2010-08-20. "In this study, egg yolks were infected at the surface of the yolk (vitelline membrane) to determine the percentage of yolk contamination (a measure used to determine egg contamination resistance, with numbers lower than 95% indicating increasing resistance): --Overall, the frequency of penetration of *Salmonella Enteritidis* into the yolk contents of eggs from individual lines of hens ranged from 30 to 58% and the mean concentration of *Salmonella Enteritidis* in yolk contents after incubation ranged from 0.8 to 2.0 log10 cfu/mL.--"

[40]. Jaeger, Gerald (Jul-Aug 2009). "Contamination of eggs of laying hens with S. Enteritidis". Veterinary Survey (Tierärztliche Umschau) 64 (7–8): 344–348. Retrieved 2010-08-20. "The migration of the bacterium into the nutritionally rich yolk is constrained by the lysozyme loaded vitelline membrane, and would need warm enough storage conditions within days and weeks. The high concentration on of antibodies of the yolk does not inhibit the *Salmonella* multiplication. Only seldom does transovarian contamination of the developing eggs with S. Enteritidis make this bacterium occur in laid eggs, because of the bactericidal efficacy of the antimicrobial peptides"

[41]. Humphrey, T.J.; A. Whitehead, A. H. L. Gawler, A. Henley and B. Rowe (1991). "Numbers of *Salmonella enteritidis* in the contents of naturally contaminated hens' eggs". Epidemiology and Infection 106 (3): 489–496. doi:10.1017/S0950268800067546. PMC 2271858. PMID 2050203. Retrieved 2010-08-19. "Over 5700 hens eggs from 15 flocks naturally infected with *Salmonella enteritidis* were examined individually for the presence of the organism in either egg contents or on shells. Thirty-two eggs (0·6%) were positive in the contents. In the majority, levels of contamination were low."

[42]. Gast, Richard; Rupa Guraya, Jean Guard, Peter Holt, Randle Moore (March 2007). "Colonization of specific regions of the reproductive tract and deposition at different locations inside eggs laid by hens infected with *Salmonella Enteritidis* or *Salmonella* Heidelberg". Journal of Avian Diseases 51 (1): 40–44. PMID 17461265. Retrieved 2010-08-20. "when hens are artificially infected with unrealistically large doses (according to the author): --In the present study, groups of laying hens were experimentally infected with large oral doses of *Salmonella* Heidelberg, *Salmonella Enteritidis* phage type 13a, or *Salmonella Enteritidis* phage type 14b. For all of these isolates, the overall frequency of ovarian colonization (34.0%) was significantly higher than the frequency of recovery from either the upper (22.9%) or lower (18.1%) regions of the oviduct. No significant differences were observed between the frequencies of *Salmonella* isolation from egg yolk and albumen (4.0% and 3.3%, respectively)--."

[43]. Cummings, PL; Sorvillo F, Kuo T (November 2010). "Salmonellosis-related mortality in the United States, 1990-2006". Foodborne pathogens and disease 7 (11): 1393–9. doi:10.1089/fpd.2010.0588. PMID 20617938.

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[45]. Feinberg School > Nutrition > Nutrition Fact Sheet: Lipids". Northwestern University. Archived from the original on 2011-07-20. Retrieved August 24, 2009.

[46]. Nutrition Data - 100g Chicken Breast

[47]. Nutrition Data - 100g Lean Skirt Steak

[48]. US Meat and Poultry Is Widely Contaminated With Drug-Resistant Staph Bacteria

[49]. J. Akbarmehr, Isolation of Salmonella spp. from poultry (ostrich, pigeon, and chicken) and detection of their hilA gene by PCR method.

[50]. Garrity, G. M., Brenner, D. J., Krieg, N. R., & Staley, J. T. (Eds.). (2005.). *Bergey's Manual of Systematic Bacteriology.* (2nd Ed.). New York: Springer.

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