UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Veterinária

Dissertação

Perfis de DNA de *Salmonella* spp. isoladas de produtos de frango e fezes de frango e humanas

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Dedico este trabalho à minha
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“Foi o tempo que dedicaste à tua rosa
que fez tua rosa tão importante.”

Antoine de Saint Exupéry

Resumo

TEJADA, Talita Schneid. **Perfis de DNA de *Salmonella* spp. isoladas de produtos de frango e fezes de frango e humanas.** 2013. 64f. Dissertação (Mestrado) – Programa de Pós-Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

Salmonella é um dos principais agentes causadores de doenças transmitidas por alimentos e os produtos a base de frango tem destaque importante neste contexto, podendo servir de veículo desse micro-organismo. O presente trabalho teve como objetivo apresentar dados quanto à propagação de *Salmonella* na cadeia avícola, verificar a ocorrência de *Salmonella* e de suas diferentes sorotipos isoladas de fezes de frango, produtos de frango e fezes humanas, bem como para verificar a similaridade entre os perfis de DNA de *Salmonella* isolados no extremo sul do Brasil. Foi realizada uma revisão bibliográfica discorrendo sobre *Salmonella* na cadeia aviária e paralelamente foi desenvolvido um projeto, no qual foram analisadas 600 amostras (200 de carne de frango, 200 de fezes de frango e 200 de fezes de humanos), quanto à presença de *Salmonella*. Os perfis de DNA das cepas isoladas foram obtidos em PFGE e REP-PCR. O micro-organismo foi isolado de 16 amostras, sendo 8 (8/200 – 4%) de produtos de frango, 4 (4/200 – 2%) de fezes de frango e 4 (4/200 – 2%) de fezes de humanos. Observou-se que, tanto em carne de frango como fezes de frango, o sorotipo predominante foi Schwazengrund, seguido de Mbandaka. Em humanos, predominou *S.* Panama. Foi constatado que de cepas com genótipos indistinguíveis estavam presentes tanto em fezes de frango como produtos de frango, sugerindo que a contaminação do frango no aviário permaneceu no produto processado. Em humanos, as cepas isoladas foram indistinguíveis entre si sugerindo que tenha ocorrido um surto, no entanto, os sorotipos isolados não foram os mesmos dos isolados de frango, o que sugere outra fonte de contaminação.

Palavras-chave: Perfil molecular. Genotipagem. *Salmonella* Schwazengrund. *Salmonella* Mbandaka. *Salmonella* Panama.

Abstract

TEJADA, Talita Schneid. **DNA profiles of *Salmonella* spp. isolated from chicken products and chicken and human stool.** 2013. 64p. Master's Thesis. Veterinary Graduate Program. Federal University of Pelotas, Pelotas.

Salmonella is one of the main causative agents of foodborne diseases, and chicken-based products play a prominent role in this context, serving as vehicles to the microorganism. The present study aimed to provide data on the *Salmonella* spread in the poultry chain, check the occurrence of *Salmonella* and its different serotypes isolated from chicken stool, chicken products and human stool, as well as to verify the similarity between DNA profiles of *Samonella* isolated. Literature on the occurrence of *Salmonella* in the poultry chain was reviewed; parallel to it, a project in which 600 samples (200 chicken meat, 200 chicken stool and 200 human stool samples) were analyzed for *Salmonella* presence was developed. DNA profiles of isolated strains were obtained by PFGE and REP-PCR. The microorganism was isolated from 16 samples, 8 (8/200 – 4%) from chicken products, 4 of which (4/200 – 2%) from chicken stool and 4 (4/200 – 2%) from human stool. *Salmonella* serotype Schwarzengrund was found to prevail both in chicken meat and chicken stool, followed by serotype Mbandaka, whereas serotype Panama prevailed in humans. Strains with indistinguishable genotypes were found to be present both in chicken stool and chicken products, suggesting that the chicken contamination on the farm remained in the processed product. In humans, the isolated strains were indistinguishable between one another, suggesting an outbreak occurrence; however, the isolated serotypes in humans were not the same as those in chickens, which is probably related to different contamination sources.

Keywords: Molecular profile. Genotyping. *Salmonella* Schwazengrund. *Salmonella* Mbandaka. *Salmonella* Panama.

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Lista de Tabelas

Lista de Abreviaturas

BHI	*Brain Heart Infusion Broth*
BPW	*Buffered Peptone Water*
^{0}C	Graus Celsius
CDC	*Centers for Disease Control and Prevention*
CF	Carne de Frango
DNA	Ácido Desoxirribonucléico
FAPERGS	Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul
FBD	*Foodborne Diseases*
FDA	*Food and Drug Administration*
FIOCRUZ	Fundação Osvaldo Cruz
FF	Fezes de Frango
FH	Fezes de Humano
kb	Kilobase
L	Litro
min	Minuto
mL	Mililitro
pb	Pares de base
%	Percentual
PCR	*Polimerase Chain Reaction*
PFGE	*Pulsed-Field Gel Electrophoresis*
pH	Potencial Hidrogeniônico
µg	Micrograma
µL	Microlitro
REP-PCR	*Repetitive Extragenic Palindromic sequence-based PCR*
s	Segundo
TSI	*Triple Sugar Iron*
WHO	*World Health Organization*

Sumário

1 INTRODUÇÃO GERAL

O Brasil é o terceiro maior produtor mundial de aves, segundo a União Brasileira de Avicultura - UBABEF, sendo superado apenas pelos Estados Unidos da América e China, todavia, é o maior exportador de carne de frango. Dentre os países que consomem carne de frango, o Brasil se encontra em quarto lugar, no entanto, se compararmos o consumo de carne bovina e avícola, observamos um crescente aumento no consumo de carne de frango, principalmente pela qualidade do produto e preços mais acessíveis (UBABEF, 2012).

As toxi-infecções alimentares, causadas pelo consumo de alimentos ou água contaminados com micro-organismos patogênicos ou suas toxinas, são uma preocupação tanto no meio científico como na indústria alimentícia (RAHMAN et al., 2013). As salmoneloses em aves ocorrem no mundo inteiro e resultam em severas perdas econômicas na avicultura devido à alta mortalidade, baixa produtividade e custos elevados no tratamento, erradicação e controle (BERCHIERI JÚNIOR & FREITAS NETO, 2009).

A contaminação dos produtos avícolas, além dos prejuízos econômicos, trás também graves consequências para a saúde pública (SINGH, 2010). Produtos a base de frango estão entre os alimentos de origem animal de maior importância na transmissão de doenças transmitidas por alimentos, principalmente devido ao frango ser um veiculador comum de salmonelose para humanos (D'AOUST, 2001). A ocorrência desta patologia é subestimada, principalmente em países subdesenvolvidos e em desenvolvimento, porém, nos Estados Unidos da América, são reportados anualmente em torno de 42.000 casos (CDC, 2012). Conhecer a epidemiologia da *Salmonella* na cadeia avícola, definindo as fontes de infecção, transmissão e disseminação, é importante para estabelecer medidas de controle e diminuir a ocorrência da salmonelose.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Apresentar dados quanto à propagação da *Salmonella* na cadeia avícola.

2.2 OBJETIVOS ESPECÍFICOS

- Verificar a ocorrência de *Salmonella* e seus diferentes sorotipos em fezes de frango no extremo sul do Brasil;
- Verificar a ocorrência de *Salmonella* e seus diferentes sorotipos em fezes de humanos no extremo sul do Brasil;
- Verificar a ocorrência de *Salmonella* e seus diferentes sorotipos em produtos de frango no extremo sul do Brasil;
- Verificar a similaridade entre os perfis de DNA de *Salmonella* isoladas de fezes de frango, produtos de frango e fezes de humanos no extremo sul do Brasil.

3 ARTIGOS

3.1 Artigo 1

Salmonella na cadeia avícola

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Salmonella na cadeia avícola

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RESUMO

Os surtos de infecção alimentar por *Salmonella*, na maioria das vezes, estão relacionados ao consumo de produtos cárneos, em especial carne de frango e seus derivados. Na cadeia de produção e processamento de aves, desde a origem do frango até a mesa do consumidor, existem vários pontos que podem ser fontes de contaminação por *Salmonella*. O objetivo deste trabalho é apresentar dados quanto à propagação da *Salmonella* na cadeia avícola. A complexa epidemiologia da *Salmonella* na cadeia de produção de aves envolve transmissão vertical, desencadeando o nascimento de pintos infectados, e horizontal, que ocorre geralmente por via oro-fecal, a partir da ingestão de água e alimentos contaminados. A infecção pode ocorrer também a partir de aves de reposição, roedores, aves silvestres e outros animais portadores. A carne e subprodutos podem sofrer contaminação durante o transporte, o abate, o processamento, ou mesmo durante o preparo do frango para consumo. Para garantir a segurança dos consumidores são necessários cuidados específicos com as aves no ambiente onde são criadas, além de medidas rigorosas quanto às condições higiênico-sanitárias durante o abate.

Palavras-chave: *Salmonella*, frangos, produtos de frango, doenças transmitidas por alimentos.

ABSTRACT

The outbreaks of food-borne disease caused by *Salmonella* in most cases are related to the meat products, especially poultry and their products. There are several points that can be sources of *Salmonella* contamination along the chicken production and processing, from the origin of the chicken to the consumer's table. The objective of this paper is to present data on the spread of *Salmonella* in chicken meat production chain. The complex epidemiology of *Salmonella* in chicken production involves vertical transmission, causing the birth of infected chicks, and horizontal, which usually occurs by oral-fecal way, through the ingestion of contaminated food and water. Infection can also occur from poultry replacement, rodents, wild birds and other animals carrying. The meat and by-products can be contaminated during transport, slaughter, processing, or even during the preparation of chicken for consumption. To ensure consumer safety are needed special care with the chickens in the environment where they are created, and rigorous measures regarding hygienic-sanitary conditions during the slaughter.

Keywords: *Salmonella*, poultry, chicken, food-borne disease.

As toxi-infecções alimentares sempre foram uma preocupação na indústria alimentícia. As bactérias do gênero *Salmonella* são consideradas, juntamente com *Campylobacter*, as maiores causadoras de doenças transmitidas por alimentos no mundo (CDC, 2011). A estimativa feita pela FOOD AND DRUG ADMINISTRATION (2009) é de que ocorram de 2

a 4 milhões de casos de salmonelose anualmente nos Estados Unidos, dos quais aproximadamente 40.000 confirmados como de origem alimentar (CDC, 2012).

Os surtos de infecção alimentar por *Salmonella*, na maioria das vezes, estão relacionados ao consumo de produtos cárneos, em especial carne de frango e seus derivados (ZONGO et al., 2010). A prevalência, no mundo, em carne de frango e derivados é bastante variável, havendo trabalhos que relatam valores de até 39% (RIBEIRO et al., 2007; YILDIRIM et al., 2011). *Salmonella* é facilmente disseminada, sendo importante para o controle da salmonelose identificar e eliminar os fatores que favorecem a multiplicação deste micro-organismo. Na cadeia de produção e processamento de aves, desde a origem do frango até a mesa do consumidor, existem vários pontos que podem ser fontes de contaminação por *Salmonella*. O objetivo deste trabalho foi apresentar dados quanto à propagação da *Salmonella* na cadeia avícola.

A patogenicidade de *Salmonella* depende do sorotipo, da cepa, da susceptibilidade e da idade das aves (DERACHE et al., 2009). Atualmente, são conhecidos mais de 2.500 sorotipos de *Salmonella*, porém, apenas alguns estão associados a infecções em humanos e animais (WHO, 2006). Os sorotipos Pullorum e Gallinarum são importantes na produção avícola, causando as doenças pulorose e febre tifóide, respectivamente, responsáveis por grandes perdas econômicas decorrentes da diminuição da conversão alimentar, debilidade do sistema imune e lesões em vísceras (SHAH et al., 2005). Os sorotipos Enteritidis e Typhimurium são de ocorrência frequente nos Estados Unidos, sendo o sorotipo Enteritidis um dos mais reportados no mundo como causa de toxi-infecções alimentares em seres humanos, através do consumo, principalmente, de produtos alimentares de origem avícola, como carne, ovos e seus derivados (CDC, 2010; STERZO et al., 2008). Segundo BERTHELOT-HÉRAULT et al. (2003), *Salmonella* Enteritidis é capaz de colonizar o trato gastrointestinal de aves domésticas, geralmente produzindo um estado de portador

crônico assintomático, exceto em aves muito jovens. O indivíduo portador contribui para a persistência do micro-organismo no ambiente e, consequentemente, para a disseminação da enfermidade.

A complexa epidemiologia da *Salmonella* na cadeia de produção de aves envolve a transmissão vertical, desencadeando o nascimento de pintos infectados (STERZO et al., 2008), os quais poderão ou não desenvolver a doença. A transmissão vertical da *Salmonella* em aves domésticas inicia pela contaminação do ovo diretamente na sua formação no trato reprodutivo ou por penetração através da casca ao passar pela cloaca contaminada com fezes (HOWARD et al., 2012; MICHAILIDIS et al., 2011). SINGH et al. (2010), em trabalho realizado na Índia, investigaram a ocorrência de *Salmonella* em ovos comerciais e observaram que 4,82% das amostras testadas estavam contaminadas com o micro-organismo. No Brasil, MEDEIROS et al. (2011) observaram a presença de *Salmonella* em 63% dos ovos comerciais analisados. Ao infectar galinhas poedeiras com uma cepa de *Salmonella* Enteritidis, GAST & BEARD (1990) observaram diminuição na produção de ovos e alta ocorrência de ovos contaminados, porém a infecção intestinal persistente do micro-organismo foi observada em um pequeno número de galinhas por curto período de tempo. A transmissão horizontal ocorre geralmente por via oro-fecal, a partir da ingestão de água e alimentos contaminados com fezes (BONI et al., 2011). SURESH et al. (2011) e MARIN et al. (2011) observaram elevada prevalência de *Salmonella* em amostras ambientais de aviário, como poeira, cama e fezes.

A infecção pode ocorrer também a partir de aves de reposição, devido à introdução de pintos contaminados (PERDONCINI et al., 2011). Roedores, aves silvestres e outros animais também favorecem a introdução e permanência da bactéria em propriedades avícolas. ROSE et al. (2000) relataram que o risco da ocorrência de *Salmonella*, após a descontaminação da granja, aumenta se houver roedores e grande tráfego de caminhões na área. CHERNAKI-LEFFER et al. (2002) observaram que insetos, como o cascudinho (*Alphitobius diaperinus*),

podem servir como veículo de *Salmonella*, considerando seu livre deslocamento na natureza. A partir da análise conjunta de vários fatores suspeitos de levarem à persistência da *Salmonella* em granjas avícolas, NAMATA et al. (2009) concluíram que os fatores mais importantes estavam relacionados à interação de trabalhadores com outras aves ou pessoas que tivessem contato com aves domésticas ou silvestres.

Durante o transporte, o abate e o processamento dos frangos, pode haver contaminação da carne e subprodutos, devido à presença de *Salmonella* nos intestinos, pele e penas dos animais, ou mesmo de outras fontes, como utensílios e mãos dos manipuladores. Segundo MENDES (2001), períodos prolongados de jejum podem afetar o pH do intestino favorecendo o crescimento populacional de *Salmonella* e de outros micro-organismos patogênicos, aumentando o risco de rompimento das vísceras e contaminação da carcaça no abatedouro. BUNCIC & SOFOS (2012) argumentam que o princípio fundamental para o controle da contaminação durante o abate é baseada em processos sanitários e de higiene, de forma que a escolha das tecnologias e operações individuais dos processos sejam abordadas com o objetivo principal de minimizar a carga microbiana no produto final. A escaldagem, extração de cloaca, abertura do abdômen e evisceração exercem papel fundamental na distribuição microbiana na carcaça de frango durante o processamento. Na escaldagem, os frangos são submersos em um tanque com água aquecida entre 50 e 60ºC, temperatura suficiente para a eliminação da maioria dos micro-organismos. No entanto, a eliminação pode não ser total, quando a carga microbiana for muito alta (CANSIAN et al., 2005). CORTEZ et al. (2006) isolaram *Salmonella* de amostras de fezes, penas, vísceras, água de escaldagem, de *chiller* e de lavagem, sendo Enteritidis e Typhimurium os sorotipos mais encontrados.

O sistema de pré-resfriamento das carcaças, realizado em duas etapas com imersão em água gelada, tem o objetivo de promover o resfriamento e reidratação. Nesta etapa, é esperada uma redução da carga bacteriana devido à remoção de sujidades (CANSIAN et al., 2005). Por

outro lado, pode ocorrer contaminação cruzada das carcaças, justificada, segundo LOPES et al. (2007), pelo uso de velocidades excessivas nas linhas de abate, equipamentos desregulados, desuniformidade no tamanho das aves, temperaturas inadequadas no *pré-chiller* e *chiller* e cloração deficiente da água.

Os equipamentos e utensílios, de uma forma geral, estão relacionados à contaminação cruzada, agindo como veículos de propagação do micro-organismo dentro da indústria. Os manipuladores podem servir de fonte de contaminação, principalmente em casos de higiene precária (VÖN RÜCKERT et al., 2009). A ocorrência de *Salmonella* foi relatada por KUSUMANINGRUM et al. (2004) em 45% das mãos dos operadores e em 35% das tábuas de corte após voluntários terem cortado em pedaços carcaças de frango contaminadas experimentalmente. Como prevenção, a antissepsia de mãos e a desinfecção de utensílios são fundamentais (MORETRO et al., 2012).

A última etapa da cadeia em que é possível a eliminação de *Salmonella* do alimento contaminado é durante o preparo do frango para consumo. Nesse sentido, devem ser evitados alimentos crus ou mal-passados, sendo suficiente o cozimento a 71ºC para a inativação da *Salmonella* (BUCHERet al., 2008).

Apesar dos avanços tecnológicos, a *Salmonella* ainda está presente ao longo da cadeia aviária. Para garantir a segurança dos consumidores, são necessários cuidados específicos com as aves no ambiente onde são criadas, além de medidas rigorosas quanto às condições higiênico-sanitárias durante o abate.

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3.2 Artigo 2

DNA profiles of *Salmonella* ssp. isolated from chicken products and chicken and human stool

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ABSTRACT

Salmonella spp. genus have been isolated from different kinds of food and are accountable for outbreaks of foodborne illnesses in humans. This study aimed to verify the similarities between the DNA profiles of *Salmonella* isolated from chicken stool, chicken products and human stool in southern Brazil. Six hundred samples were collected (200 chicken product, 200 broiler chicken stool and 200 human stool) and analyzed each sample for the presence of *Salmonella*. The strains were tested biochemically and serologically. The characteristics strains confirmed by the Polymerase Chain Reaction, after the DNA profiles were analyzed by PFGE and REP-PCR. *Salmonella* was isolated from 16 out of 600 analyzed samples, 8 of which (8/200 – 4%) from chicken products, 4 (4/200 – 2%) from chicken stool and 4 (4/200 – 2%) from human stool. We found the highest occurrence of serotype Schwarzengrund, followed by Mbandaka and Panama. It was found that strains whose genotypes were

indistinguishable by the molecular methods used in the study, suggesting that the source of contamination may have had a same origin.

Keywords: *Salmonella,* chicken stool, human stool, chicken products.

1. Introduction

For years professionals in the food safety field have tried to prevent the growth of deteriorating and pathogenic microorganisms in food. Bacteria of the *Salmonella* spp. genus have been isolated from various kinds of food and are accountable for outbreaks of foodborne diseases (FBD) in humans, being the most frequent cause of diarrhea in the United States, where around 42,000 cases are reported annually (CDC, 2012). In Brazil, 8,663 cases of FBD were reported to the Ministry of Health between 2000 and 2011, and *Salmonella* was the main etiological agent identified (BRASIL, 2011).

Among products of animal origin, chicken-based ones are the most important in *Salmonella* transmission to humans (Aslam et al., 2012). *Salmonella* prevalence in chicken meat and chicken products varies, with studies reporting values of up to 39% in the world (Ribeiro et al., 2007; Thakur et al., 2013). The source of contamination of chicken products often originates in the chicken aviaries, where *Salmonella* occurrence is also high, (Le Bouquin et al. (2010) report the prevalence of 8.6% in broiler chicken flocks.

Samonella is easily spread, so it is important to understand the epidemiology of the microorganism to prevent toxinfections in humans by the consumption of contaminated food (Tahergorabi et al., 2012). The identification of *Salmonella* clones in animals, food and humans is important for the knowledge of the salmonellosis epidemiology dynamics in the food chain, which is the indispensable basis to draw up effective plans for disease control.

Pulse-Field Gel Electrophoresis (PFGE) has been an important tool in the investigation of FBD outbreaks, having been used for the rapid detection of related cases and implicated food (Favieret et al., 2013) through phenotyping, i.e. the estimation of the genetic distances between strains of the same species. Also, the discrimination ability of this technique makes it a relevant contamination source traceability tool within a food chain, enabling the isolation of strains of the same species from different sites involved in food processing (Ribolet, 2006).

Another molecular method used in the genetic distance characterization of bacterial species is the amplification of repetitive extragenic regions (Repetitive Extragenic Palindromic Sequence-based Polymerase Chain Reaction – REP-PCR) dispersed in their genomes, which provides distinct patterns of amplified bands. REP-PCR is a simple and fast method which, despite showing good reproducibility, has moderate discriminatory power (Tyler et al., 1997) as compared to PFGE. The latter molecular technique is currently thought to be the "gold standard" for subtyping many pathogenic organisms (CDC, 2011).

This study aimed to verify the similarities between the DNA profiles of *Salmonella* isolated from chicken stool, chicken products and human stool in southern Brazil.

2. Material and Methods

2.1 Sample

Six hundred samples were collected: 200 chicken product, 200 broiler chicken stool and 200 human stool samples between August 2011 and July 2012.

The chicken product samples (40 drumstick/thigh, 40 wing, 40 dorsal, 40 ground meat and 40 liver samples) were obtained chilled at retail sales from southern Brazil, kept in their original wrappings, packed in cool boxes and immediately sent to the laboratory. Products of

16 different brands were collected, one of which (brand A) from a chicken slaughterhouse headquartered in the studied area, from which 80 samples were collected, and the other 15 (bands B to P) from products marketed in this area, of which 120 samples were analyzed.

The chicken stool were collected with swabs at the time of slaughter from the contents of the large intestine, which was sectioned longitudinally immediately after the cecal region by using sterile surgical scissors. The chickens were proceeding from 40 different chicken farms. Five random batch samples were collected from each aviary. The material was keeping in tubes with 10 mL BPW to send to laboratory.

Human stool samples were obtained with the help of clinical analysis laboratories in the area under study, which kindly provided the material for analysis. Swab sample were collected from collection containers sent to the laboratories and keeping in tubes with 10 mL BPW to send to laboratory. All patients whose stool were included in this study showed abdominal discomfort and had been instructed by their personal physician to collect fecal samples for analysis.

2.1 Isolation and identification

The chicken product samples, depending on each case, either 25 g or the whole sample was placed in sterile plastic bags containing 100 mL Buffered Peptone Water (BPW, Acumedia, Lansing, Michigan) and massaged for 5 minutes. The resulting suspension was drained and used as a pre-enrichment step for *Salmonella* presence detection, in compliance with US Food and Drug Administration (FDA) recommendations (Andrews & Hammack, 2007). And, the chicken and humans stool were then incubated in test tubes added with 10 mL BPW for pre-enrichment as well as other *Salmonella* research procedures, as mentioned earlier.

Isolates which proved to be *Salmonella* compatible by biochemical and serological tests were tested by the Polymerase Chain Reaction (PCR) as suggested by Malorny et al. (2003) for identification confirmation. The primer used were 5'GTGAAATTATCGCCACGTTCGGGCAA and 5'TCATCGCACCGTCAAAGGAACC witch target to the gene *invA*. DNA was extracted as recommended by Sambrook & Russel (2001) from a pure culture in Brain Heart Infusion Broth (BHI, Acumedia) at 37º C. 12,5 µL Master Mix (Promega, Madison, Wisconsin, USA), 1 µL of each primer, 5 µL DNA and 5,5 µL deionized water to complete the reaction volume were used. Amplification was performed in a TC-3000 Thermal Cycler (Techne, Staffordshire, UK) following these steps: initial denaturation at 95º C for 1 minute, followed by 38 denaturation cycles at 95º C for 30 seconds, annealing of primers at 64º C for 30 seconds, extension at 72º C for 30 seconds, and final extension at 72º C for 4 minutes. For the amplification analysis, the technique of gel electrophoresis in 1% agarose (Panreac Química SA, Barcelona, Spain) was used. GeneRuler™ 1 kb DNA ladder (Fermentas, Vinius, Lithuania) was used as marker. After GelRed™ (Uniscience, São Paulo, São Paulo, Brazil) strain, gels were scanned using L-PIX EX (Loccus biotecnologia, Cotia, São Paulo, Brazil).

After PCR confirmation, the strains were referred to the Department of Bacteriology of the Enterobacteria Laboratory of the Oswaldo Cruz Foundation (FIOCRUZ, Manguinhos, Rio de Janeiro) for serotype identification.

The 24h cultures in BHI broth at 37º C were added with 20% glycerol and frozen at -70º C for stock maintenance. The strains were incubated in BHI at 37º C for recovery.

2.2 Molecular profiles

Isolates were analyzed by PFGE, following the PulseNet PFGE Manual protocol suggested by the Centers for Disease Control and Prevention (CDC, 2009). For DNA fragment cleavage, *XbaI* restriction endonuclease (New England Biolabs™ Inc., Beverly, MA, USA) was used. To determine phenotype, PFGE was performed by using gel in a CHEF-DR® II Pulsed Field Electrophoresis System apparatus (Bio-Rad Laboratories, Hercules, CA, USA) added with 1% agarose Grau Pulsed Field Cromossomal (Bio Agency Laboratories, SP, BR). The gel was subsequently stained with ethidium bromide (100µg/mL) and visualized with UV light.

REP-PCR was performed in agreement with the methodology described by Versalovicet et al. (1994). Aliquots of 25 µL solutions containing 12,5 µL Master Mix, 8 µL deionized water, 2 µL (GTG)5 5'GTGGTGGTGGTGGTG3' primer and 2 µL previously extracted DNA were submitted to PCR amplification, which was performed in a TC-3000 thermal cycler following the steps: initial denaturation at 94º C for 5 minutes, followed by 30 denaturation cycles at 95º C for 30 seconds, annealing of primers at 45º C for 60 seconds, extension at 60º C for 5 minutes, and final extension at 60º C for 16 minutes. For amplification analysis, the technique of electrophoresis in 2% agarose gel was used; GeneRuler™ 1 kb DNA ladder was used as marker; for band visualization, GelRed under ultraviolet light was used.

The PFGE and REP-PCR patterns were interpreted in accordance with criteria suggested by Tenoveret et al. (1995) by using the classifications: indistinguishable (no different bands), closely related (2 to 3 distinct bands), possibly related (4 to 6 distinct bands) and different (over 7 distinct bands).

3. Results and Discussion

Salmonella was isolated from 16 out of 600 analyzed samples, 8 of which (8/200 – 4%) from chicken products, 4 (4/200 – 2%) from chicken stool and 4 (4/200 – 2%) from human stool.

Among the 40 chicken farms analyzed, the bacterium was isolated from 4 chicken stool samples from 3 farms. Of the 8 contaminated chicken product samples, 3 (1,5%) were liver, 3 (1,5%) drumstick/thigh, 1 (0,5%) wing and 1 (0,5%) dorsal samples. Three brands (A, L and N) marketed products which contained *Salmonella*. Brand A had 5 (6% - 5/80) contaminated samples, brand L, 2 (11% - 2/18) and brand N, 1 (33% - 1/3).

Ribeiro et al (2007), differently from the results of our study but also in southern Brazil, collected 61 chicken products (wings, whole legs, boneless breasts, and backs) at a processing plant and observed that 39,3% of the samples had been contaminated with *Salmonella*. Conversely, other studies in northeastern Brazil (Duarte et al., 2009; Oliveira et al., 2006) obtained similar results to those in this study. Duarte et al. (2009) analyzed 260 chicken carcasses bought from five different processing plants, and found that 9,6% of the carcasses were positive for *Salmonella*. Oliveira et al. (2006), in a study in which 63 chicken carcasses from two processing plants and two supermarkets were collected, observed an 11,8% carcass positivity for the microorganism.

All strains phenotypically characterized as *Salmonella* showed the *invA* gene, a highly preserved DNA region of this genus which can confirm the identification at a molecular level, as proposed by Malorny et al. (2003).

Four distinct serotypes were identified (Table 1). Two phenotypically distinct colonies (FF02 and FF03) were obtained from chicken stool samples (one of the colonies showed typical biochemical characteristics, and the other completely acidified TSI agar), and were

confirmed as *Salmonella* by serology and PCR. These isolates were identified as being from 2 distinct serotypes; it was also found that the same chicken harbored serotypes Scharzengrund (FF02) and Mabandaka (FF03) simultaneously.

Serotypes Enteritidis and Typhimurium have been reported in other research studies (Suresh et al., 2011; Thakur et al., 2013) as being the most commonly isolated in chicken; this study, however, did not identify any isolates of these serotypes in chicken meat or stool, having found the highest occurrence of serotype Schwarzengrund, followed by Mbandaka. Other studies have also reported a higher prevalence of serotypes other than Enteritidis and Thyphimurium, such as that of Aslam et al. (2012), in Canada, who analyzed ground beef samples, and the study of Le Bouquin et al. (2010), in Frace, who included broiler chicken samples; these authors found a higher prevalence of serotype Hadar, not observed in this study.

In a study conducted by the Department of Bacteriology of the Oswaldo Cruz Foundation, Hofer et al. (1997) reported that serotype Mbandaka belongs to a common, yet infrequent, *Salmonella* group; serotype Schwarzengrund, on the other hand, is thought to belong to a rare, or accidental, *Salmonella* group, according to occurrence levels in the 1962 – 1991 period. Nevertheless, as previously mentioned, the predominant serotype both in chicken stool (3 isolates from 2 aviaries) and meat (6 isolates) in this study was Schwarzengrund. Boni et al. (2011) reported that this serotype was also the most frequently isolated in a study done at a slaughterhouse in Mato Grosso do Sul State, Brazil, between August 2005 and December 2006; serotype Schwarzengrund, however, was not isolated in the aviaries of this region, leading to the conclusion that contamination had occurred at the slaughterhouse rather than on the chicken farm. Chen et al. (2010) reported a high prevalence of this serotype in raw chicken meat (30,5%) in Taiwan.

The 9 isolated *Salmonella* Schwarzengrund strains were submitted to genotyping by PFGE with *XbaI* restriction enzyme, and no differences between band patterns were observed (Figure 1A). However, differences between some strains were observed in the REP-PCR technique (Figure 1B). The CF03, CF04, CF06, CF07 and CF08 strains isolated from chicken meat are indistinguishable from one another; notwithstanding, they are closely related to the FF01 and FF02 strains, which come from chicken stool and are indistinguishable from each other and possibly related with CF05 (chicken meat) and are different to FF04 from chicken stool.

CF03 and CF06 strains were isolated from a commercial brand (brand L), which suggests that contamination occurred from the same source, whether the farm the chicken came from or the slaughterhouse where brand L is processed. In the case of the strains isolated from brand A products – CF04, CF07 and CF08 – the results point to a common contamination source. Moreover, the correlation between the strains isolated from brand A products with FF01 and FF02 chicken stool isolate strains may be due to the fact that the farms supply chickens to the brand slaughterhouse. This suggests that the contamination of these foods originated on the chicken farms.

The lack of differentiation among most Schwarzengrund seroptype strains by PFGE may have been due to the low cut-off frequency of *XbaI* restriction endonucleases, resulting in 5 – 6 bands, whereas REP-PCR was able to amplify 6 bands. The genomic DNA of this serotype has approximately 52,9% G+C (NCBI, 2012) and the use of restriction endonucleases which can recognize adenine and thymine-rich DNA sites, as is the case of the enzyme used in this study, which recognizes the 5'TCTAGA site, may have originated a smaller number of fragments than expected, not discriminating strains satisfactorily as predicted. According to Acuña et al. (2002), there is no restriction endonuclease that might be considered the most discriminating for the PFGE technique, once the number of profiles

obtained for each enzyme varies in accordance with individual genetic characteristics of each strain. Aarestrup et al. (2007), upon studying the detection of *Salmonella* Schwarzengrund clones with PFGE in chicken food and human isolates, showed the transmission of the microorganism from food to man, as opposed to this study, where isolates from chicken were not found to be related to those from humans.

Salmonella Mbandaka was found in 2 chicken stool samples from two different origins and 1 chicken product sample (wing). Other studies (Suresh et al., 2011; Hue et al., 2011) also report a low occurrence of this serotype. Upon analyzing different cuts of broiler chickens, these authors noticed that this serotype was one of the least common in southern India, yet it was present in different chicken parts, in addition to having been found in a cage environmental sample. In France, Hue et al. (2011) again registered the low occurrence of this serotype, having identified only one isolate from 425 chicken carcasses from a slaughterhouse. In this study, serotype Mbandaka corresponded to 17% (3/17) of the isolated samples.

PFGE (Figure 2A) and REP (Figure 2B) results show that the CF02 and FF03 strains are indistinguishable from one another, suggesting that the source of contamination is the same. The FF05 strain, which is not related to CF02 and CF03 strains, probably had a different origin from the latter. Hoszowski & Wasyl (Poland, 2001) reported that biotyping, susceptibility profile to antimicrobials and plasmid profiles were not enough to differentiate the *S.* Mbandaka strains analyzed, and only genomic macrorestriction did prove to be an efficient method in epidemiological studies for this serotype. However, this study observed that the discriminatory power of REP-PCR was comparable to those presented by PFGE for this serotype, with the advantage that the former technique is less expensive and faster than PFGE.

242 *Salmonella enterica* rugosa was isolated from 1 chicken meat sample. Alcocer et al.
243 (2006), upon evaluating 25 *Salmonella* strains obtained from chicken carcasses from 4
244 slaughterhouses in Paraná State, Brazil, found only one rugosa strain. Other authors have
245 reported the isolation of rugosa strains in chicken stool in Brazil (Salles et al., 2008; Andreatti
246 Filho et al., 2009). Nevertheless, rugosa strains were isolated from chicken meat rather than
247 chicken stool in this study. Though not proved, the possibility of this meat having been
248 previously contaminated by chicken stool cannot be ruled out.

249 Children up to 5 years of age are more affected by salmonellosis than older individuals
250 (CDC, 2012). However, in a study reporting an outbreak in Sao Paulo, Brazil, Matsuoka et al.
251 (2004) observed that the average age of the affected people was 36,5 years. In the present
252 study the age of patients with *Salmonella* which was isolated from stool samples varied from
253 9 months to 40 years, without sex predominance.

254 In human stool samples, 3 Panama and 1 Typhimurium serotype strains were isolated.
255 These serotypes have been known to cause gastroenteritis in humans, both in Brazil
256 (Fernandes et al., 2006) and in other countries (Soto et al., 2001; Tsai et al., 2007). However,
257 data provided by the Center of Disease Control and Prevention (CDC, 2012) connecting 12
258 *Salmonella* outbreaks of animal origin in humans in 2012 did not include serotypes Panama
259 and Typhimurium. In this study, serotype Panama strain showed band profiles
260 indistinguishable (Figure 3) from one another, which is suggestive of an outbreak occurrence,
261 insofar as the three samples from patients in the same location were collected on the same
262 day.

263

264 **4. Conclusions**

265

Salmonella is present in broiler chickens in southern Brazil, as well as in chicken products available for consumption, which is a consumer health risk. *S.* Schwarzengrund was the predominant serotype in this study, followed by Mbandaka, both in aviaries and chicken products, and whereas in humans the most frequently isolated serotype was Panama. It was found that strains whose genotypes indistinguishable by the molecular methods used in the study occurred in chicken stool and chicken products, suggesting that the chicken contamination on the farm remained in the processed product. These data warn of need for greater hygienic and sanitary care by processing plants regarding the control of undesirable microorganisms and greater care in the aviary biosecurity in order to minimize the risk of contamination of the final product.

Strains that were isolated in humans were of different serotypes from those found in chicken products, and this suggests that the source of contamination may have had a different origin. The fact that the human strains indistinguishable by the techniques used suggests the occurrence of an outbreak. Likewise, other salmonellosis cases and outbreaks can occur without being reported to authorities, which eventually contributes to an underestimation of the disease records in humans in Brazil.

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449 Table 1 – *Salmonella* serotype isolated from analyzed samples.

Serotype	Chicken meat	Chicken stool	Human stool
Rugosa	CF01	-	-
Mbandaka	CF02	FF03*; FF05	-
Schwarzengrund	CF03; CF04; CF05; CF06; CF07; CF08	FF01; FF02*;FF04	-
Typhimurium	-	-	FH01
Panama	-	-	FH02; FH03; FH04

450 * Strains isolated from the same chicken.
451

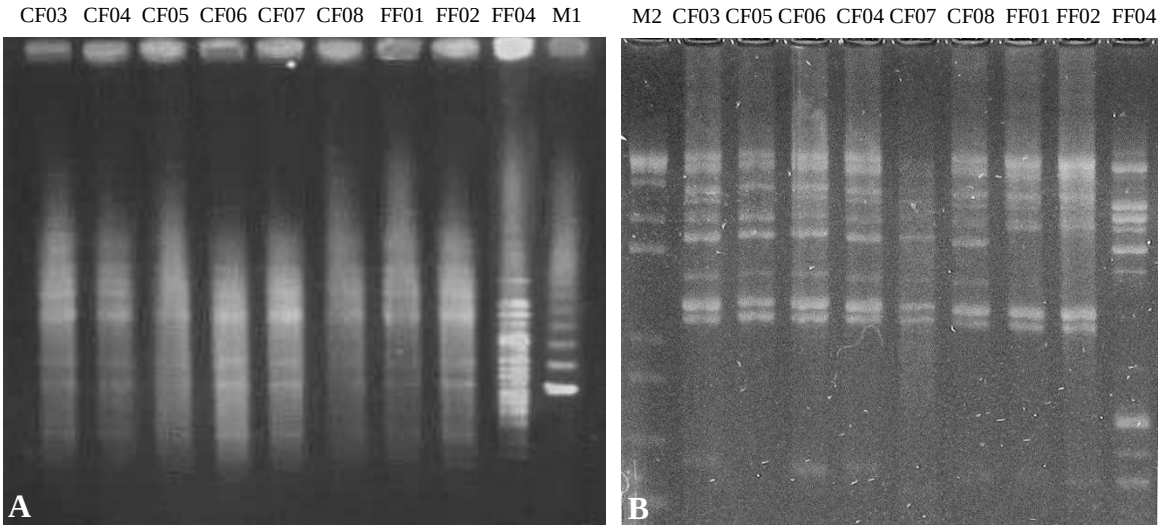

Figure 1: Photographs of PFGE (A) and REP-PCR (B) electrophoresis gels with nine isolate band profiles isolated of *Salmonella* Schwarzengrund. CF03: isolate from brand A chicken drumstick; CF04: isolate from brand B chicken drumstick; CF05: isolate from brand C liver; CF06: isolate from brand A liver; CF07: isolate from brand B liver; CF08: isolate from brand B back; FF01: isolate from G02 farm chicken stool; FF02 and FF04: isolate from farm G15 chicken stool; M1: DNA Size Standards – Lamba Ladder; M2: GeneRuler™ 1 kb DNA ladder.

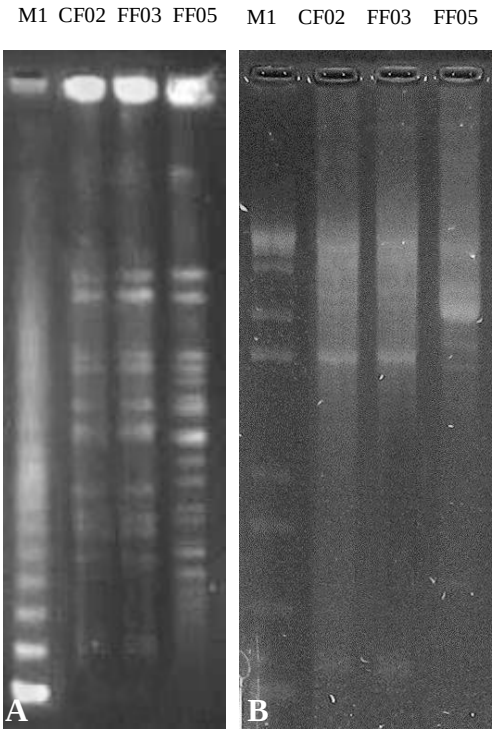

Figure 2: Photographs of PFGE (A) and REC-PCR (B) electrophoresis gels with three isolate band profiles isolated of *Salmonella* Mbandaka. M1: DNA Size Standards – Lamba Ladder; M2: GeneRuler™ 1 kb DNA ladder; CF02: isolate from brand B chicken wing; FF03 and FF05: isolate from chicken stool.

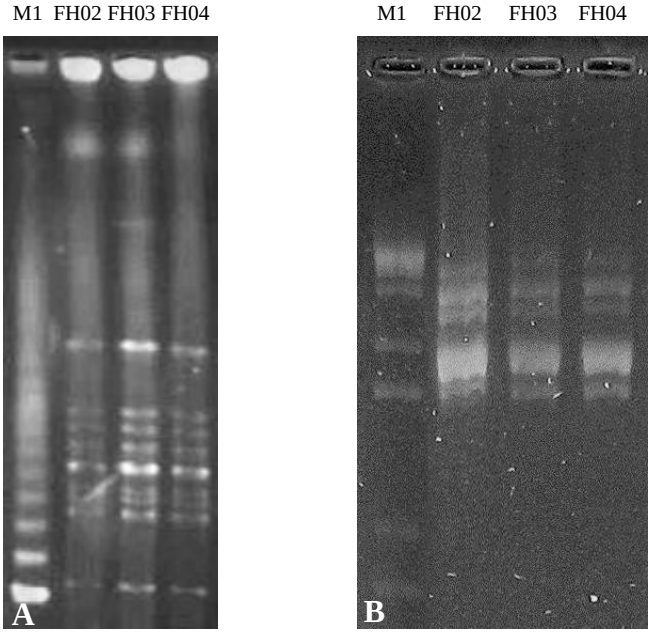

Figure 3: Photographs of PFGE (A) and REC-PCR (B) electrophoresis gels with three isolate band profiles isolated of *Salmonella* Panama. M1: DNA Size Standards – Lamba Ladder; M2: GeneRuler™ 1 kb DNA ladder; FH02, FH03 e FH04: isolate from human stool.

4 CONCLUSÃO GERAL

Este estudo demonstra que apesar dos avanços tecnológicos, a *Salmonella* ainda está presente ao longo da cadeia aviária oferecendo risco a saúde do consumidor.

Dentre os sorotipos isolados de carne e fezes de frango, *S.* Schwazengrund é o sorotipo predominante, seguido de Mbandaka. Em humanos, o sorotipo predominante é Panama.

A detecção de cepas com genótipos indistinguíveis, presentes tanto em fezes de frango como em produtos de frango, sugere que a contaminação dos frangos no aviário permaneceu no produto processado. Além dos cuidados com as aves no ambiente onde são criadas, também são necessárias medidas rigorosas quanto às condições higiênico-sanitárias durante o abate para garantir a segurança dos consumidores.

As cepas isoladas de humanos foram indistinguíveis pelas técnicas moleculares, o que é sugestivo de que tenha ocorrido um surto. No entanto, por serem distintas das encontradas em produtos de frango, possivelmente a fonte de contaminação tenha sido de outra origem. Estes resultados são indicativos da falta de identificação e notificação de casos e surtos de salmonelose, os quais podem passar desapercebidos e a doença ser subestimada.

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ANEXOS

ANEXO 1

Submissão do Artigo 1

Manuscrito 127_12 LIPOA x

Arquivos do I. biologico <arquivos@biologico.sp.gov.br> 03/12/12

para mim

Prezado Autor,
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Informamos que seu trabalho recebeu o número **Arq. 127/12**. Qualquer esclarecimento que desejar solicitar favor informar este número.
Para ciência dos autores, informamos que o trâmite (da entrada ao aceite) de um manuscrito nesta revista está demandando uma média de 12 meses.
Atenciosamente,
Silvia R. Galleti
Editor Chefe

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ANEXO 2

Normas para submissão do Artigo 1

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A Revista Arquivos do Instituto Biológico, publicada trimestralmente, aceita, para submissão, artigos originais de pesquisa científica em sanidade animal e vegetal voltados ao agronegócio e suas implicações no agroambiente, incluindo nesse escopo a qualidade e a segurança alimentar. Aceita, também, artigos sobre pragas sinantrópicas. Todos os trabalhos devem se enquadrar nas normas redatoriais.

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Resumo: deverá apresentar concisamente o objetivo do trabalho, material e métodos e conclusões, em um único parágrafo. Não ultrapassar 250 palavras.

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Abstract: apresentar uma tradução para o inglês, do título do trabalho e do resumo. A seguir, relacionar também em inglês (ou espanhol) as mesmas palavras-chave (key words, palabras-clave) já citadas. Não ultrapassar 250 palavras.

Introdução: descrever a natureza e o objetivo do trabalho, sua relação com outras pesquisas no contexto do conhecimento existente e a justificativa da pesquisa feita.

Material e Métodos: apresentar descrição breve, porém suficiente para permitir uma repetição do trabalho. Técnicas e processos já publicados, exceto quando modificados, deverão ser apenas citados. Nomes científicos de espécies, bem como drogas, deverão ser citados de acordo com regras e padrões internacionais.

Resultados: apresentá-los acompanhado de tabelas e/ou figuras, quando necessário. As tabelas e figuras devem ser inseridas após as referências.

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Tabelas e Figuras: incluir título claro e conciso que possibilite o seu entendimento sem consultas ao texto. As tabelas não deverão conter linhas verticais. No texto, use a palavra abreviada (ex.: Fig. 3). As figuras devem estar no formato jpg (fotos) ou gif (gráficos e esquemas) e com tamanho inferior a 500 Kb. As figuras originais ou com maior resolução poderão ser solicitadas após o aceite. Devem ser enviadas em arquivos individuais e nomeadas de acordo com o número da figura. Exemplos: Fig1.gif, Fig2.jpg.

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3. Short Communications
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5. Book Reviews

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Reviews of topics in veterinary bacteriology, mycology and virology should provide short, readable, well- referenced, up-to-date overviews of current, emerging, or neglected subjects in the discipline. Syntheses of information from diverse sources, providing clarification of areas of confusion or uncertainty, are especially desirable. It is anticipated that these reviews will provide overviews of important topics to the benefit of curious-but-busy readers of Veterinary Microbiology.

Reviews should carry titles which are creative and provocative, but nonetheless descriptive, and emphasize current status and future directions of research. Historical vignettes are useful in setting the stage for addressing important contemporary questions, but should not ordinarily be the basis for an article. Manuscripts may include controversial views, if presented in balanced fashion and supported by evidence; informed speculation is welcome. For reasons of credibility, it is preferred that reviews be written by authors from more than one research center.

Authors may find it useful to contact the Reviews Editor J. Glenn Songer (jgsonger@iastate.edu), perhaps with an outline of a proposed review, before submitting. Articles should be about fifteen pages of double-spaced type, supported by illustrative material. Figures are welcomed and articles should not have more than 50 references. Manuscripts should be submitted through the EES electronic submission system, taking care to clearly indicate that it is a review article.

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Acknowledgements
All contributors who do not meet the criteria for authorship as defined above should be listed in an acknowledgements section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support. Authors should disclose whether they had any writing assistance and identify the entity that paid for this assistance.

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