Detection of Salmonella spp. antigens in Tolima poultry products by Western Blot*

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ABSTRACT: ***Salmonella*** spp., is a Gram-negative bacterium transmitted to human by consumption of contaminated water and food consumption, mainly poultry products like eggs and chicken meat. The aim of this study was to implement the Western Blot technic to detect the presence of antigenic proteins of ***Salmonella*** spp., in chicken carcasses and egg surface and if possible to compare it with the traditional microbiological isolation. A total of 18 chicken carcasses and 18 eggs were collected from the 13 communes and 5 marketplaces of Ibagué city. A chicken carcass and egg surface washes were obtained and an aliquot from each one was processed by using standard international guidelines ISO 6579-1:2017 for bacterial isolation. Another aliquot was centrifuged and the pellet separated by polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred in to a nitrocellulose membrane by Western blot. Primary antibody was an in-house rabbit polyclonal anti-***Salmonella*** enteritidis antiserum and the secondary antibody was a goat anti-rabbit IgG conjugated with alkaline phosphatase. The reaction of the two antibodies was detected with the addition of the BCIP-NBT enzyme substrate and the image recorded with a digital camera. Antigenic bands of ***Salmonella*** spp. of 10, 15, 17 and 40 kDa and 10, 17, 25, 37 and 75 kDa were detected in 15 out of 18 (83,3 %) and 4 out of 18 (22,2 %) samples from chicken carcasses and egg surface respectively. A total of 4 out of 36 samples were positive to ***Salmonella*** spp., by microbiological isolation. It is concluded that the SDS-PAGE and Western blot technic can successfully detect***Salmonella*** antigens in chicken carcasses and egg surface and it may constitute a valuable complementary tool for the detection of this microorganism in poultry products.

Key Words: antibodies, bacteria, diagnosis, electrophoresis, isolation, proteins.

Detección de antígenos de *Salmonella* spp., en productos avícolas del Tolima, por la técnica de Western Blot

RESUMEN: ***Salmonella*** spp., es una bacteria Gram-negativa transmitida por el agua o alimentos contaminados como los productos de origen aviar. El objetivo de este estudio fue implementar la técnica de Western Blot para la detección de proteínas antigénicas de ***Salmonella*** spp., en canales de pollo y superficie de huevo y comparar dicha técnica con el cultivo microbiológico. Un total de 18 muestras de canales de pollo y 18 de huevo fueron colectadas de las 13 comunas y 5 plazas de mercado de la ciudad de Ibagué. Se obtuvo un lavado de la superficie del pollo y otro de la superficie del huevo; una alícuota fue procesada mediante el protocolo estándar internacional ISO para el aislamiento bacteriano. Otra alícuota fue centrifugada y separada mediante electroforesis en gel de poliacrilamida (SDS-PAGE). Las proteínas separadas fueron transferidas a una membrana de nitrocelulosa a través de la técnica de Western Blot. El anticuerpo primario fue un antisuero policlonal anti-***Salmonella*** Enteritidis generado en conejo y el anticuerpo secundario, una IgG comercial de cabra anti-conejo conjugada con la enzima fosfatasa alcalina. La reacción de los dos anticuerpos fue detectada mediante la adición del substrato BCIP-NBT. Se detectaron bandas antigénicas de***Salmonella*** spp., de 10, 15, 17 y 40 kDa y 10, 17, 25, 37 y 75 kDa en 15 de 18 (83,3 %) y 4 de 18 (22,2 %) muestras de canales de pollo y superficie de huevo respectivamente. Un total de 4 de las 36 muestras fueron positivas a ***Salmonella***spp., por aislamiento microbiológico. Se concluye que la técnica de SDS-PAGE y Western Blot puede detectar exitosamente antígenos de ***Salmonella*** en canales de pollo y superficie de huevos y constituye una herramienta valiosa complementaria en el diagnóstico de la bacteria en productos avícolas.

Palabras clave: aislamiento microbiológico, anticuerpos, diagnóstico, electroforesis, proteínas.

Introduction

The genus ***Salmonella*** comprises 2 species: ***Salmonella enterica*** and***Salmonella bongori***, although a third species named ***Salmonella subterranea***(Shelobolina et al., 2004), was recognized in 2005 and it might be incorporated in to the CDC system (Su & Chiu, 2007). ***Salmonella enterica*** is further subdivided into six subspecies that include ***enterica, salamae, arizonae, diarizonae, houtenae*** and ***indica*** and currently there are more than 2610 ***Salmonella***serotypes (Yoshida et al., 2016), that are motile, facultative intracellular, non-lactose fermenting, Gram-negative microorganisms (Bopp et al., 2015; Quinn et al., 2001).

Salmonella is classified according to the different antigens present in the bacterial cell wall, the O antigen is known as somatic antigens and the H antigen is constituted by polymerized subunits of flagellin, whereas the virulence-associated antigen expressed in the surface of some ***Salmonella*** strains is called Vi or K antigen (Parra et al., 2002). The use of a number of commercial antisera directed to some of those surface antigens of ***Salmonella*** constitutes a universal subtyping method called Serotyping.

Several ***Salmonella enterica*** serotypes may cause infections in humans and animals; however, S. enteritidis and S. typhimurium are widely distributed and represent the main serovars associated with disease in human (Hendriksen et al., 2004; Canals et al., 2011). ***Salmonella*** is a foodborne pathogen that is usually transmitted to humans by ingestion of contaminated water and food (Velge et al., 2012), including raw chicken (WHO, 2008) and eggs (Perez et al., 2008), which are the most common sources of infection.

Foodborne illness occurs at a rate of one million, nineteen thousands hospitalizations and 380 deaths each year in the United States (CDC, 2016), meanwhile in the European Union, over 100000 human cases are reported each year (EFSA, 2017). Globally, it is estimated that 93,8 million cases of gastroenteritis and 155000 deaths are caused by ***Salmonella*** species each year (Majowicz et al., 2010), even though the number of cases is high, 60-80 % of all ***Salmonella*** infections are not recognized as part of a known outbreak and they usually are classified as sporadic cases, or are not properly diagnosed at all (WHO, 2014).

Isolation and identification of ***Salmonella*** species is a long process that requires microbiological culture and agglutination tests (Ospina-Florez et al., 2014; Van der Zee, 1994). ***Salmonella*** isolation solely is a time-consuming process that include steps like pre-enrichment, enrichment in selective agars and sub-cultivation of the samples in a variety of culture mediums (Hendriksen, 2003). To overcome those limitations, the Poultry Research Group of the University of Tolima has implemented molecular techniques to speed up the identification of ***Salmonella*** present in poultry products, among them are the use of Polymerase Chain Reaction (PCR) (Rodriguez et al., 2015), Multiplex PCR (Mogollon et al., 2016), Multilocus Sequence Typing (MLST) and Pulse Field Gel Electrophoresis (PFGE) (Rodriguez et al., 2015). The Western Blot is a fast and sensitive technic for detection and characterization of proteins based on their recognition by antibodies (de la Fuente et al., 2007). The technique has been used to detect different kinds of ***Salmonella*** proteins (Humphries et al., 2005). In this pilot study the Western Blot technique was implemented to detect the presence of antigenic proteins of ***Salmonella*** spp., in raw chicken and shell eggs, as an alternative diagnostic tool compared to the traditional microbiological isolation.

Materials and Methods

Sample collection

A total of 36 samples (18 chicken meat samples and 18 eggs) were collected from stores and supermarkets located in the 13 communes and 5 marketplaces of Ibagué city during May-November of 2016. Each sample consisted of one whole chicken leg weighing about 300 gr and 3 eggs collected at each store. The eggs were processed in a pool, thus obtaining 2 samples from each store or supermarket. The samples were packed in sterile plastic bags kept at 4 ºC and transported to the Laboratory of Veterinary Diagnosis for processing within 3 hours.

Salmonella Isolation

All samples were processed according to standard international guidelines ISO 6579-1:2017. Briefly chicken meat samples were incubated in buffered peptone water during 24 hours at 37 ºC for pre-enrichment, and subsequently an aliquot of the peptone water was seeded in Tetrathionate Broth (Muller-Kauffmann) and Rappaport Vassiliadis and incubated at 37 ºC and 42 ºC respectively for the colony selective enrichment. Later, bacterial cells were seeded on McConkey and XLT4 (Xylose Lysine Tergitol) agar. Compatible colonies were seeded in Rambach agar and confirmed as ***Salmonella*** spp., by an agglutination test with Poli AI + Vi antibodies (Difco® 222641). Positive colonies were confirmed biochemically using API® 20E gallery (Biomereux, France). The surface of eggs was rubbed in buffered peptone water and the surface wash was used as the sample and followed the same culture protocol.

Protein extraction and SDS-PAGE

To extract and separate proteins from chicken meat and egg washes, the meat pieces and pooled eggs were rinsed in peptone water for 10 minutes until a surface wash was obtained. The wash was centrifuged at 1500 rpm and the precipitated material was resuspended in PBS. A total of 50 µL of each sample was mixed with 50 µL of 2 x Laemmli buffer and boiled at 95 ºC for 5 minutes. Later samples were centrifuged at 12000 rpm at 4 ºC for 4 minutes and 20 µL of each supernatant were taken and seeded in a 15 % polyacrylamide gel. Electrophoresis was conducted at 80V for 30 minutes (prerunning) and 110V 102 minutes (running). The gel was stained with Coomasie brilliant blue overnight and discolored in decolorizing agent (water, methanol and acetic acid). Later, photographic record was taken.

Western Blotting

The proteins separated in a second polyacrylamide gel were transferred into a nitrocellulose membrane by using a Trans-Blot SD semi-dry cell (Bio-Rad) transfer equipment. The proteins from the gel were transferred at 15V for 90 minutes. After

transference, the nitrocellulose membrane was blocked with BSA (TBS-Triton + BSA) for 1 hour, and washed twice for 10 minutes with TBS-T. After blocking the membrane was incubated with the primary rabbit anti-***Salmonella*** antibody at a dilution of 1:500 for 1 hour and then washed with TBS-T twice for 10 minutes. Subsequently the membrane was incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:15000 for 1 hour. Reaction of the two antibodies was detected with the addition of 5-Bromo-4-Chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP-NBT) enzyme substrate (Sigma-Aldrich, St. Louis, MO, USA).

Production of an Anti-Salmonella polyclonal antiserum

A pure culture of ***Salmonella*** Enteritidis isolated from laying hen farms (Rodriguez et al., 2015) was inactivated with 1 % buffered formalin and incubated at room temperature overnight. Inactivated bacterial cells were collected by centrifugation, washed and re-suspended on PBS. Bacterial suspension was homogenized with Complete Freund's adjuvant (CFA) (Sigma Aldrich, St Louis, MO, USA) and inoculated into four New Zealand female rabbits via subcutaneous and intramuscular routes at different sites, with periodic booster inoculations. On day 52 post inoculation (p.i) total blood was collected from the marginal vein of the ear. The blood was left at 4 ºC overnight and, the serum was decanted and collected after centrifugation at 2500 rpm during 15 minutes at 4 ºC. Cross-reactive antibodies present in the hyperimmune serum were removed by incubation with ***Escherichia Coli*** ATCCC 25922 for 30 minutes. Bacterial cells with bound cross-reactive antibodies were removed by centrifugation at 12000 rpm and this immunoadsorption protocol was repeated twice before the serum could be used in western blot.

Results and Discussion

Western Blot analysis and molecular techniques based on immunological methods for detection of specific proteins of ***Salmonella*** spp. had been used for a long time (Cooper & Thorns, 1996; Fadl et al., 2002; Findik et al., 2010), and it has been described as an effective identification method based on potent antigenic protein detection (Maripandi & Al-Salamah, 2010). Based on those advantages of Western Blot compared to the time-consuming microbiological isolation, this study shows for the first time that this technique was effective in detecting the presence of***Salmonella*** in poultry products in the Tolima Department.

Surface washes of the chicken carcasses and eggs were processed by SDS-PAGE and Western Blot and positivity to ***Salmonella*** antigens were considered when consistent

bands on the nitrocellulose membrane were apparent. A total of 15 out of 18 (83,3 %) chicken carcasses were positive for ***Salmonella*** antigens, indicating the presence of ***Salmonella*** in chicken carcasses. This result is higher than that obtained by microbiological isolation, where only 2 out of 18 (11,1 %) samples were positive for ***Salmonella*** isolation. In addition, a total 4 out of 18 (22,2 %) egg surface wash samples were found positive for ***Salmonella*** antigens even though***Salmonella*** was not detected (0 %) by microbiological isolation of those samples (Table 1).

Table 1. Frequency of Salmonella positive poultry products by Western blotting and Microbiological isolation

	Western blot				Microbiological Isolation			
	Chicken meat	%	Egg surface	%	Chicken meat	%	Egg surface	%
Positive	15	83,33	4	22,22	2	11,11	0	0
Negative	3	16,67	14	77,78	16	88,89	18	100
Total samples	18		18		18		18	

The results indicate a high sensitivity of the Western Blot technique to detect***Salmonella*** in poultry samples when compared to microbiological culture. Thus, we proposes Western Blot as an alternative diagnosis tool for epidemiologic evaluation/detection of ***Salmonella*** spp., in poultry products such as chicken meat and eggs that could speed up the identification of contaminated or ***Salmonella***-free poultry products.

We generated a polyclonal antibody in rabbits that was immuno absorbed with ***E. coli*** cells due to presence of cross-reactive antibodies. This step seems to be very important to remove weak and non-specific antigen-antibody interactions responsible for undesirable background. Cross reactive antibodies are normally present when using polyclonal antisera and even with the use of monoclonal antibodies there could be non-specific immunoglobulins (Keller et al., 1993; Lee et al., 1989; Thorns et al., 1994). The cross-reactivity is in part due to the high similarity and amino acid identity between antigenic proteins from Enterobacteria, that could be as high as 73-82 % between ***E. coli***, ***Citrobacter*** and ***Enterobacter***species (Yeh et al., 2016; Nhan et al., 2011). However, to reduce the potential background caused by cross-reactive antibodies, we performed various immunoadsorption with total proteins from ***E. coli*** (Sambrook, 2001), that allowed us to detect a reduced number but specific antigenic proteins from ***Salmonella***.

The SDS-PAGE and Western Blot assay identified antigenic protein bands of different molecular mass in both kinds of samples. In chicken carcasses protein bands of approximately 10, 15, 17, and 40 kDa were commonly detected (Figure 1B), whereas in the egg surface wash samples, bands of 10, 17, 25, 37, 75 kDa were frequently

observed. Although the SDS-PAGE and Western blot assay implemented in this study could not identify the protein represented by each immunoreactive band, recent studies have reported that several ***Salmonella***antigenic proteins corresponds to outer membrane proteins (OMPs) (Maripandi & Al-Salamah, 2010). Thus, implementing standard protocols for outer membrane proteins extraction could be useful to identify particular antigens of ***Salmonella*** with high immunoreactivity with the polyclonal IgG.

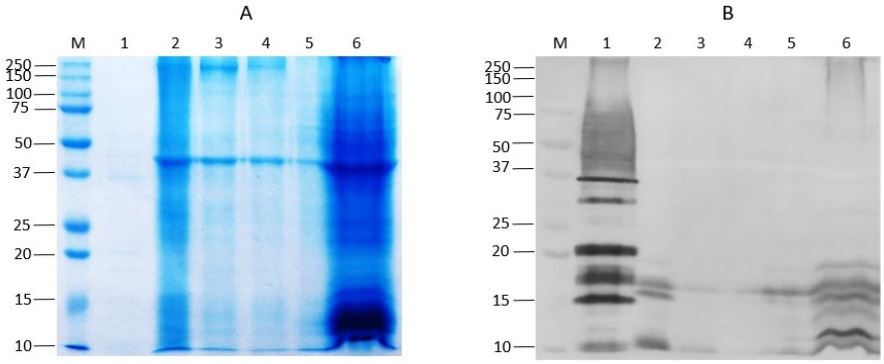

Figure 1. Detection of ***Salmonella*** spp. in poultry products by SDS-PAGE and Western Blot assay. A. Representative SDS-PAGE of total proteins from chicken carcasses and egg's washes stained with Coomasie brilliant blue. B.Western Blot of total protein extracted from chicken carcasses and egg's washes that shows detection of ***Salmonella*** spp., antigens M: Protein marker; Lane 1: Positive control of***Salmonella*** enteritidis ATCC 13076***;*** Lanes 2 to 6: Samples of chicken carcasses.

Differences in the intensity of the bands obtained in the Western Blot assay have been described previously and it is related with the genetic variability, strain, pathogenic and metabolic features of ***Salmonella*** spp. (El-Fakar & Rabie, 2009; Helmuth et al., 1985). Interestingly, the electrophoretic profile of the proteins extracted from different chicken carcasses (Figure 1A) and egg surface wash samples, produced some proteins bands visualized by SDS-PAGE that matched with antigenic protein bands detected by Western Blot, however other immunoreactive bands were not visualized by SDS-PAGE, and may suggest a different nature of those antigens, perhaps polysaccharides complexes. In addition, the amount of proteins from the positive control needed to be detected by Western Blot was significantly less than the amount of protein from chicken meat and egg wash samples. This may indicate that although the ***Salmonella*** present in each sample was significantly low it could be consistently detected by WB.

Proteins with molecular masses of 16 kDa (Koski et al., 1989), 25 kDa (Cordova, 1998) 37 and 40 kDa (Helmuth et al., 1985; Singh et al., 2007; Verdugo-Rodriguez et al., 2009), 75 kDa (Khan et al., 2003) and a fimbria of 17 kDa (Collinson et al., 1991) in different ***Salmonella*** serotypes have been identified as antigenic proteins. Future studies should use more robust techniques such as 2-dimensional gel electrophoresis (2D) to identify the antigenic proteins of ***Salmonella*** that could be explored as

immunodominant antigens. In addition, recombinant DNA technology can also be used to generate recombinant proteins to assess their antigenicity and immunoreactivity with our polyclonal anti-***Salmonella*** antibody that help us to identify the most dominant antigenic proteins.

The SDS-PAGE and Western blot assay implemented in this study showed more effectivity when compared to microbiological isolation. The Western blot assay could detect a very low number of antigenic proteins from ***Salmonella*** spp., that may indicate a low number of bacterial cells in the wash samples and they were not detected by microbiological isolation (Gonzalez et al., 2014), however the specificity of Western Blot technique was lower than microbiological isolation when the hyperimmune sera was used without immunoadsorption. Additional studies are necessary to evaluate the possibility to increase the specificity of the Western Blot technique to detect ***Salmonella*** in poultry products, for example if an antigenic protein of ***Salmonella*** is identified to have a very low amino acid identity with the proteins of other Enterobacteria, it could be possible to produce a polyclonal antiserum specifically to that antigen. This procedure could optimize the Western blot technique as an alternative diagnostic tool for ***Salmonella*** in poultry products and food and other products and eventually reduce the transmission of ***Salmonella***to the consumer. Detection of antigenic proteins of ***Salmonella*** spp., in poultry products by Western blot may indicate the presence of live bacteria in those products but it may also possible that only bacterial cell debris were present. In this regard the low frequency of ***Salmonella*** isolation could give support to the second possibility, however, in the pursuit for high quality and safety poultry products, in the future the establishment of minimal levels of bacterial proteins in food products, as a reflect of bacterial contamination could be established and novel and more sensitive techniques developed.

Finally, the total time required for the SDS-PAGE and Western blot assay was approximately 8 hours, which was less time consuming than the standard microbiological isolation technique to detect ***Salmonella*** spp.

Conclusions

This study has implemented an additional molecular technique, the SDS-PAGE and Western Blot to detect ***Salmonella*** in poultry products in the Tolima region. The results, are promissory for the poultry field and need to be included in future projects. In addition, the study allowed the involvement of undergraduate students of the Veterinary Medicine program and constitutes an approximation to a novel work field of Veterinarians that allow them to use standard technologies and to visualize the upcoming technologies, their use in research and epidemiological investigations and

academy. The regional poultry industry should pay attention to these efforts directed to understand the epidemiology of ***Salmonella*** and the safety of poultry products and the education of Veterinary students in research focused on avian health. These results without a doubt will contribute to improve the quality and hygienic conditions of poultry products before human consumption.

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