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Detection of β - Lactamases Genes in some Salmonella Isolated from Poultry in Khartoum North, Sudan

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Abstract

The occurrence of extended spectrum beta lactamases genes in bacteria (ESBLs) is one of the problems that facing the world now in treatment of bacterial infection. This study was conducted to detect CTX-M, SHV, and TEM genes in ESBLs producing *Salmonella gallinarum* and *Salmonella pullorum*. All *Salmonella* strains were isolated from samples collected from poultry farms located in Khartoum north and identified with conventional methods. Bacterial DNA was extracted from each isolate (*S.pullorum*, *S.gallinarum*) using boiling method. PCR was used to detect TEM, SHV, and CTX-M genes. The results showed that the genotypic resistance that is mediated by β-lactamases genes in *S. gallinarum* was (100%) for SHV followed by CTX-M and TEM genes both (58%) and in *S. pullorum* was (44%) for CTX-M then TEM (33%) and finally SHV genes (11%).

Keywords: Salmonella, β – Lactamases, Poultry, Sudan.

Introduction

Non-typhoidal *Salmonella* spp. have been described as major pathogens associated with food-borne gastroenteritis worldwide (Threlfall, 2002). While antibiotics are not usually recommended in cases of *Salmonella enterocolitis*, their use for therapeutic purpose becomes important when the pathogen becomes invasive as in meningitis, sepsis and bacteraemia (Threlfall, 2002). In cases of such life-threatening complications, extended-spectrum cephalosporins are usually the drug of choice (Hohmann, 2001). During the last 2 decades, extended-spectrum beta-lactamases (ESBLs) found in Gram-negative bacilli has emerged as a significant mechanism of resistance to antibiotics. The ESBLs mediate resistance to broad-spectrum cephalosporins (e.g., ceftazidine, ceftriaxone, and cefotaxime) and aztreonam. The genes encoding ESBLs are usually found on plasmids, along with genes encoding mechanisms of resistance to aminoglycosides and trimethoprim- sulfamethoxazole. Finally, the combined effect of multiple ESBLs and outer membrane protein (OMP) deficiencies may lead to resistance of ESBL-producing enteric bacteria to lactam lactamase inhibitor combinations and, occasionally, even to cephamycins and carbapenems. More than 100 genetically distinct TEM-type and SHV-

type ESBLs have now been characterized. The occurrence of resistance to the extended-spectrum beta-lactamases (ESBLs) among members of the family *Enterobacteriaceae* is a growing to be a worldwide public health problem (Bradford, 2001). The principal mechanism of resistance to the extended-spectrum beta-lactam antibiotics involves the production of ESBLs (Shahada *et al*., 2010). The ESBLs hydrolyze oxyimino-cephalosporins and monobactams, but not cephamycins and they can sometimes be inhibited by clavulanic acid (CVA) (Shahada , *et al* 2010).The AmpC type of β-lactamases on the other hand hydrolyze cephamycins and cephalosporins but are not inhibited by CVA (David, 2003).

Salmonellae have been reported to express different types and the prevalence of genes encoding for them varies from region to region (Winokur *et al*., 2001). These enzymes such as TEM (AitMhand *et al*., 2002), SHV (Baraniak *et al*., 2002), PER (Bradford *et al*., 1998), DHMA (Revathi *et al*., 1998), VEB (Villa *et al*., 2000), GES (Pitout *et al*., 2003), ACCM (Rankin *et al*., 2002), OXA (Casin *et al*., 2003) and CTX-M enzymes (Hanson *et al*., 2002).

More than 340 beta-lactamases have been described in *Salmonella* strains (Hasman *et al*., 2005) There is however a paucity of information on the genes encoding these betalactamases, despite resistance to beta-lactam drugs in Salmonella isolated from humans and food animals in developing countries(Ogunleye *et al*., 2005). Rapid spread of genes of resistance to antimicrobial agents can occur in a bacterial population and from one ecosystem to another; hence the development of resistance in one bacterial population can spread to other populations overtime through sharing and exchange of resistance genes. In a variety of interconnected ecosystems, antimicrobial agents can lead to the emergence of resistance, the reduction of microorganisms susceptible to the agents, and the drastic alterations in the biodiversity of affected ecosystems. Antimicrobial resistance is clinically relevant because 3-10% of infections can progress to life-threatening bacteraemia, particularly in young and immuno-compromised patients (Okeke *et al*., 2005).

In Ethiopia, a resistance pattern of *Salmonella* isolates from chickens indicated large proportions of strains resistant to a variety of drugs (Molla *et al*, 2003), and this has led to a shift in the antibiotics used against *Salmonella* species in Nepal from chloramphenicol and ampicillin to trimethoprim-sulfamethoxazole, fluoroquinolones and ceftriaxone (Pokharel *et al*, 2006).

The mechanisms of antimicrobial resistance are numerous including possession of additional gene by some bacteria for protection against bactericidal effects of drugs, change of their permeability to the drug in use, etc. One of the most disturbing mechanisms of resistance to drug is the production of an enzyme known as beta-lactamase by some bacteria. The beta-lactamase is responsible for the resistance of the bacteria to beta-lactam antibiotics like penicillin, cephamycins and carbapenems. These antibiotics have a common element in their molecular structure, and that is, a four-atom ring known as beta-lactum. The lactamase enzyme breaks the ring open, deactivating the molecule's antibacterial properties (Philippon *et al*, 2002).

Most of the research done in Sudan focused on the antimicrobial resistance phenotypes among *Salmonella ssp* isolated from animals. Almost no data have been published concerning the molecular bases of this resistance of Sudanese local isolate (Molla *et al*, 2003). This study was designed to screen two multidrug resistant *Salmonella* species isolated from septic poultry in Sudan – Khartoum North, 12 *S. gallinarum* and 18 *S. pullorum* for three possible genes encoding a variety of beta-lactamases enzymes responsible for resistance to some antibiotics that are still very much in use for treatments of Salmonella infection in Sudan.

This study was done to identify some genes encoding beta-lactamases capable of causing transferable resistance in animals and human, thus constituting a potential public health risk.

Material and Methods

Study design

Bacteria: Eighteen *S. pullorum* and 12 *S.gallinarum* were isolated from poultry farms in Khartoum North, Sudan. They were identified by conventional methods (Barrow & Feltham, 1993).

Resistance to Aztreonam, Imipenem and Piperacillin: The *Salmonella* isolates potentially harboring ESBLs were those with a positive phenotypic confirmatory test for ESBLs according to current National Committee for Clinical Laboratory Standards (NCCLS) criteria. To test for this positive phenotypic, 3 antibiotics were used Aztreonam, Imipenem and Piperacillin.

Bacteria were grown aerobically in breakpoint concentrations of Aztreonam, Imipenem and Piperacillin (SIGMA-ALDRICH) according to standard method (CLSI, 2009). Resistance was ascribed if flocculent growth was observed after 16 h of aerobic growth at 37°C.

A phenotypic confirmatory test was then performed by testing MICs for Aztreonam, Imipenem and Piperacillin - clavulanic acid. A threefold concentration decrease in a MIC of Aztreonam, Imipenem and Piperacillin tested in combination with clavulanic acid versus its MIC when tested alone was indicative of phenotypic confirmation of ESBL production.

DNA extraction: DNA was isolated from each of the 30 resistant *Salmonella* isolates, adding about 250 µl of bacterial culture to 750 µl of distilled water and boiled for 10 minutes. The boiling solution was centrifuged and the super was used as DNA template.

PCR amplification conditions: Three sets of primers targeted the following gene classes: TEM, SHV and CTX-M were used to amplify the respective genes from plasmid DNA.

PCR was performed in a 20 µl reactions containing 4 µl of master mix (containing dNTBs (0.4µl), Tag polymerase (0.25µl), MgCL2 (1.5µl), buffer (2.5µl)), 0.4 µl of forward primer, 0.4 µl of reverse primer, 1 µl of template (sample) and 14.2 µl of water. Convergys® td peltier thermal cycle (Germany) was used for the DNA amplification using the following PCR protocols:

For CTX-M gene: Initial denaturation at 94 °C for 5 minutes, followed by 32 cycles of denaturation at 94 °C for 40 seconds, primer annealing at 50 °C for 35 seconds and elongation at 72 °C for 50 seconds. Final extension at 72 °C for 7 minutes (Naas, *et al*, 2005).

For SHV gene: Template denaturation for 5minutes at 95 °C followed by 35 cycles of an initial denaturation step at 94 °C for 30 seconds, primer annealing

60 °C for 60 seconds, elongation at 72 °C for 1 minutes, final extension at 72 °C for 7minutes, (Rankin *et al*, 2002).

For TEM gene: Initial denaturation step at 96 °C for 15 seconds followed by 24 cycles of DNA denaturation at 96 °C for 15 seconds, primer annealing at 50 °C for 15 seconds and primer extension at 72 °C for 2 minutes. After the last cycle the products were stored at 4 °C, (Pitout *et al*, 1998). Amplified DNA products were subjected to electrophoresis using 1% (w/v) agarose gel stained with Ethidium bromide. Three sets of primers were used in characterizing β- -lactamases as in Table (1).

Table 1: Three β- lactamases gene targeted in the study, the primers oligosequences and related β-lactamases

Name	Sequence (5' -------- 3')	Product size	Related enzymes	References
CTX-R	5- ACC GCG ATA TCG TTG GT - 3¯			(Naas, *et al*,
CTX- F	5- CGC TTT GCG ATG TGC AG - 3¯		CTX-M-1-	2005).
		550 bp	CTX-M-	
			82.	
SHV-R	5- TGC TTT GTT ATT CGG GCC -3¯			(Rankin *et*
SHV- F	5- ATG CGT TAT ATT CTG TG - 3¯		SHV1-	*al*, 2002)
		753 bp	SHV63	
TEM-R	5- AGC GAT CTG TCT AT - 3¯			(Pitout *et al*,
TEM-F	5- AAA CGC TGG TGA AAG TA - 3¯	752 bp	TEM1-	2003)
			TEM190	

Results

Thirty β- lactams phenotypic resistant *Salmonella* isolates (12 of *S. gallinarum* and 18 isolates *S. pullorum)* were tested for the presence of 3 betalactamase genes. PCR results showed that 7.0 *S. gallinarum* isolates were positive to CTX-M (58%) and 8.0 *S. pullorum* isolates were positive to CTX-M (44%). For SHV genes, 12.0 isolates of *S. gallinarum* (100%) and 2.0 isolates of S. *pullorum* (11%) were positive. For TEM genes, 7.0 isolates of *S. gallinarum* (58%) and 6.0 isolates of S. *pullorum* (33%) were positive.

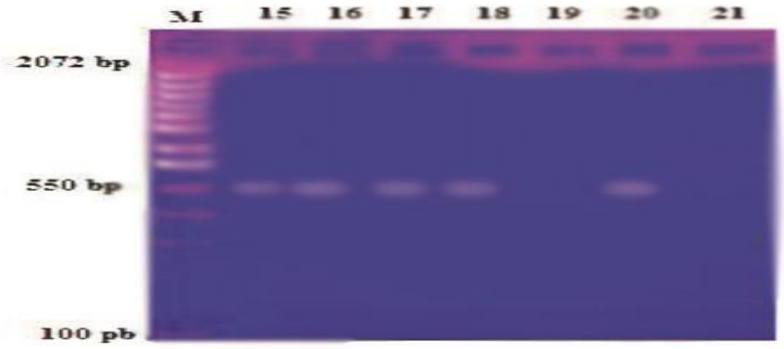

Figure 1: Positive CTX-M samples of Salmonella

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella pullorum* isolates with CTX-M specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp) , lane 1 is positive control lanes 2,3,4 and 6were positive to the CTX-M genes lanes 5and 7 were negative.

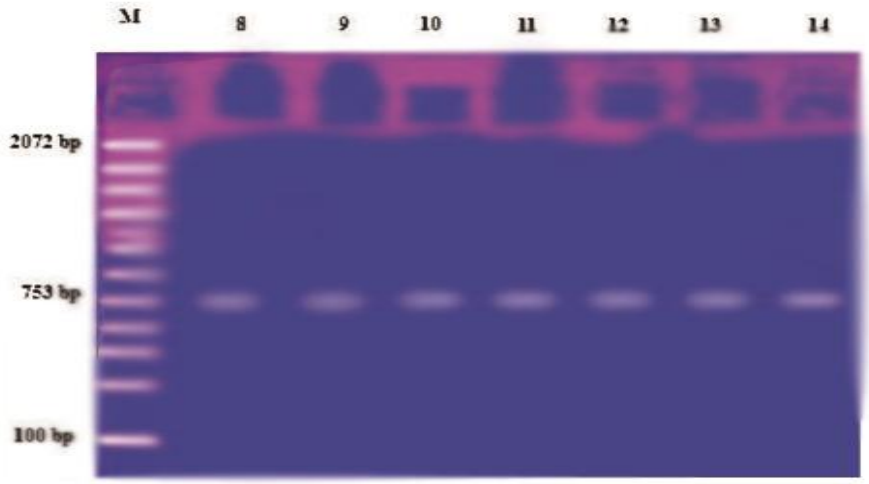

Figure 2: Positive SHV samples

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella gallinarum* isolates with SHV specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp), lane 1 is positive control lanes 2, 3,4,5,6 and 7were positive to the SHV genes.

Figure 3: Positive TEM samples

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella gallinarum* isolates with TEM specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp), lane 1 is positive control, lanes 2, 3,4,5,6 and 7 were positive to the TEM genes.

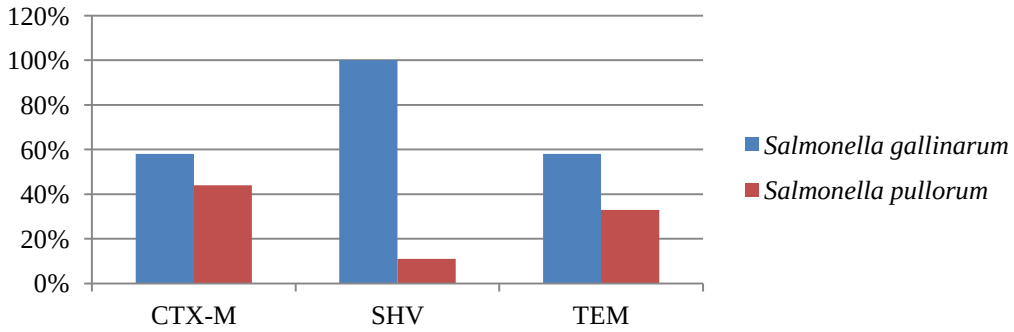

Figure 4: Genotypic Resistance Pattern of Salmonella isolates

As shown in Figure (4) the heights percentage of genes recorded was (100%) in *S. gallinarum* isolates for SHV followed by CTX-M and TEM genes both (58%), for *S. pullorum* isolates was (44%) for CTX-M then TEM (33%) and finally SHV genes (11%).

Discussion

Antibiotics have multi use in animals e.g. for treatment of infectious diseases and also they are used as growth promoters. This over use of antibiotics may result in the emergence of resistant bacteria that can be transmitted to human via the food supply. The relationship between the use of antibiotics drugs for animals and the emergence of antibacterial drugs resistant pathogenic bacteria in human is well reported (AitMhand *et al*., 2002).

Food animals are important sources of food borne pathogens. Poultry are common source of *Salmonella* for the human consumers. *Salmonella* is an important cause of food-borne gastroenteritis in human (Bouallegue *et al*., 2005).

Salmonella organisms have been reported to express varieties of extended spectrum beta-lactamases Some beta-lactamases that have been described in *Salmonella* include TEM, SHV, CTX-M, and OXA families, (Armand-Lefevre *et al*., 2003; Hanson *et al*., 2002). Sometimes some of these genes can occur in multiples in a single isolate (Armand-Lefevre *et al*., 2003; Hanson *et al*., 2002). All across the globe, there has been various reports incriminating *Salmonella* species like *S. gallinarum* and *S. pullorum* producing TEM, SHV, and CTX related beta lactamases in nosocomial infections (Yong *et al*.,2005)..

The present study demonstrated varying reactions in the use of antimicrobials against the *Salmonella* isolates from poultry. The isolates showed highly different results, however SHV encoding enzymes responsible for most of *S.gallinarum* resistance as well as *S. pullorum* resistance followed by CTX-M and TEM. A similar activity has been reported among *Salmonella* species in other countries like Turkey, Nepal and South Africa (Irajian *et al*., 2009). The enzyme has been reported to bring about resistance to Piperacillin, Ceftazidime and aztreonam as it is coded on

conjugative plasmids, transposons or integrons, genetic materials which can be spread readily (Irajian *et al*., 2009).

Since the emergence of *Salmonella* isolates harbouring extended-spectrum beta lactamases (ESBLs), it has grown to be a major public health problem worldwide (Bonnet, 2004). Resistance to third-generation Beta-lactams in *Salmonella* which often results from the production of plasmid-mediated extended-spectrum beta-lactamases (ESBLs) has been reported worldwide (Parry, 2003). In Sudan however, there are paucity of such reports both in *Salmonella* serotypes from human and food animal origin. This work thus provides an initial database for genes responsible for β- lactams resistance in *Salmonella* strains isolated from food animals from Khartoum North area. The findings in this work expose the possible health risk in terms of transfer of drug resistance from these food animal to man. Beta- lactams are still the drug of choice in treating some life threatening infections in developing countries (Naas *et al*., 2005).

It is important to monitor the emergence of resistant bacteria from food animals, such animals may be important source of these resistant bacteria which can be spread from their products directly to man, it can jeopardize success of effective treatment thus constituting a potential grave public health hazard.

Conclusion

It is concluded that there is a widespread Beta-lactamase activity in and around the poultry, causing antibiotic resistance of Salmonellae and other species of bacteria. This obvious resistance pattern observed could be due to Beta-lactamase activity which is a presently known problem of antibiotic resistance. This is a serious health implication for poultry consumption and therefore the need for the control of indiscriminate antibiotic use in poultry, a situation which encourages antibiotic resistance thus exacerbating an existing global problem of antibiotic resistance.

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تقصي جينات انزيمات البيتا لاكتاميز في بكتيريا السالمونيلا المعزولة من مزارع الدواجن في الخرطوم بحري، السودان

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المستخلص:

تعتبر الإنزيمات الممتدة الطيف واحدة من المشاكل التي تواجه العالم الآن في علاج العدوى البكتيرية. أجريت هذه الدراسة للكشف عن الجينات (سي تي إكس – أم ، شيف و تيم) في بكتريا السالمونيلا الدجاجية و السالمونيلا الفراضية المنتجة لإنزيمات بيتا لاكتميز الممتدة الطيف. تم الحصول على الأنواع البكتيرية عبر عزلها و التعرف عليها معملياً من عينات أخذت من مزارع الدواجن شمال الخرطوم – محلية بحري. تم إستخلاص الحمض النووي الديوكسي رايبوسي بتقنية الغليان وأستخدمت نظرية التفاعل التسلسلي المتعدد للتعرف على وجود جينات البيتالاكتميز تيم ، شيف و سي تي إكس – أم وقد أظهرت النتائج وجود هذه الجينات بدرجات متفاوتة حيث كانت نسبتها في السالمونيلا الدجاجية 58% لجينات سي تي إكس – أم ، 58 % تيم و 100% شيف. و عند دراسة وجود هذه الجينات في معزولات السالمونيلا الفراضية سجلت 44% لجينات سي تي إكس – أم ، 33 % تيم و 11% لجينات شيف.