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Full Length Research Paper

Development and application of an enzyme-linked immunosorbent assay (ELISA) using a soluble recombinant nucleoprotein for the detection of antibodies to avian influenza virus

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Avian influenza (AI) causes significant impact on industrial poultry farming, besides infecting a variety of vertebrates. The detection of antibodies against viral antigens by serological methods is important for the epidemiology, control and prevention of AI because their high simplicity and speed for assaying a large number of samples. Obtaining antigenic preparations used for detection of anti-avian influenza virus (AIV) antibodies usually requires complex and expensive procedures and *Escherichia coli* system expression may be an alternative. The nucleoprotein (NP) of AIV is an ideal antigen candidate because it is highly conserved across AIV strains, resulting in high cross-reactivity and immunogenicity for avian hosts. The NP gene segment was cloned and expressed from AIV isolate H4N6 in *E. coli* fused to a small ubiquitin-like modifier (SUMO) polypeptide and a poly-histidine tag, obtaining a soluble recombinant NP (rNP) containing the most important epitopes. After purification, the rNP was used as an antigen to develop an indirect rNP-enzyme-linked immunosorbent assay (ELISA) to effectively detect anti-AIV antibodies in chicken serum samples. This rNP-ELISA had high sensitivity (95%), specificity (97%), accuracy (96.7%) and agreement (k=0.88) in a comparative analysis with a commercial ELISA kit. The results suggest that rNP-ELISA offers a viable alternative to improve immunodiagnosis of AIV infection in chickens.

Key words: *Escherichia coli* system expression, small ubiquitin-like modifier (SUMO)-peptide, immunodiagnosis, poultry.

INTRODUCTION

Avian influenza (AI) is an infectious disease of domestic and wild birds that causes significant impact on the health of animals and on industrial poultry farming, besides infecting a wide variety of vertebrates, including ducks, chickens, pigs, whales, horses, and seals (Swayne and Halvorson, 2003). Different subtypes of avian influenza virus (AIV) type A cause this disease. Even though molecular techniques are efficient for the direct diagnosis of AIV, the detection of antibodies against specific viral antigens by serological methods is still considered an important tool for the epidemiology, control, and prevention of AI, because they can usually handle a large number of samples and generate results more rapidly at lower costs. The most used serological techniques are agar-gel immunodiffusion (AGID), hemagglutination-inhibition (HI), and enzyme linked immunonosorbent assay (ELISA) (OIE, 2015). ELISA is widely used because it requires smaller amounts of antigen and fewer antibody handlings than either of the other two techniques, whilst also enabling the evaluation of a larger number of samples, in a shorter period (Swayne and Halvorson, 2003; Wu et al., 2007). Antigenic preparations used for detection of anti-AIV antibodies with ELISA are usually obtained through procedures, such as viral propagation in specific-pathogen free (SPF) embryonated chicken eggs followed by purification of virus particles by ultra-centrifugation (OIE, 2015), which are complex and expensive. The expression of heterologous viral proteins is a more practical, simple and economical alternative to these techniques, as recombinant proteins tend to conserve most of the immunochemical properties of the original homologous proteins from viral particles and thus can be effectively used as antigen preparations in different immunodiagnosis methods. Indeed, as NP of AIV is highly conserved across AIV strains and present high cross-reactivity among these viruses and high immunogenicity for avian hosts, it has been expressed as recombinant protein to be used for the detection of AIV-specific antibodies by different ELISA types in chicken sera (Jin et al., 2004; Shafer et al., 1998; Upadhyay et al., 2009; Wu et al., 2007).

Escherichia coli is the most widely used microorganism for the production of recombinant proteins, due to fast growth kinetics, substantial protein production in a short time, ability to reach high cell density cultures in artificial media prepared from readily available and low-cost compounds, and great accessibility for transformation with exogenous DNA (Gopal and Kumar, 2013; Rosano and Ceccarelli, 2014; Sambrook and Russel, 2001). Despite these advantages, the *E. coli* expression system has not been more frequently used to produce AIV recombinant NP because this protein is difficult to express in this system, and must usually be recovered from the insoluble fraction, requiring several purification steps (Jin et al., 2004; Wu et al., 2007).

An approach used to circumvent this limitation is to increase the solubility of expressed recombinant proteins through the use of vectors harboring peptide and tags, such as the small ubiquitin-like modifier (SUMO) peptide (Guerrero et al., 2015; Zuo et al., 2005). Such vectors have been constructed for cloning and expression in *E. coli* systems, but to date this approach has not been used for the expression of AIV proteins.

Influenza A subtype H4N6 is one of the most prevalent subtypes isolated from a large variety of avian hosts (Hinshaw et al., 1981; Olsen et al., 2006). It is circulating around the world and identified in epidemiological surveillance studies with wild and domestic birds in Asia (Deng et al., 2013; Liu et al., 2003; Okamatsu et al., 2013), Europe (Henriques et al., 2011) and North America (Hanson et al., 2003; Scotch et al., 2014). First isolated in Czechoslovakia (1956) (Koppel et al., 1956), H4N6 AIV strains are classified as low-pathogenic avian influenza (LPAI) because animals infected with this subtype generally have the AI asymptomatic form (Olsen et al., 2006), although there are reports of disease and systemic spread in chickens infected experimentally with H4N6 in China (Liu et al., 2003). Influenza A subtype H4N6 has been isolated in Canada since 1999 from pigs with pneumonia (Karasin et al., 2000). In North America, H4N6 was the most isolated subtype in 2007 and 2008 by Wilcox et al. (2011). Kang et al. (2013) found that the predominant subtype in domestic ducks and wild birds in Korea was H4N6 and Latorre-Margalef et al. (2014) also verified that H4N6 was the major subtype found in migratory mallards in Northern Europe between 2002 and 2010.

Interestingly, multiple H4N6 AIV strains co-circulate and recombine with other influenza viruses in live poultry markets and farms (Shi et al., 2016). Since domestic pigs can support recombination of human and avian influenza viruses under natural conditions it is important to increase surveillance for this influenza virus subtype (Karasin et al., 2000).

Considering the positive effect of SUMO fusion technology in the expression of recombinant proteins in the *E. coli* system and the importance of influenza A subtype H4N6, the objectives of this study were to express the nucleoprotein gene of this AIV subtype in a soluble form and to use it as an antigen preparation in an indirect ELISA (rNP-ELISA) to detect anti-AIV antibodies in chicken serum. Additionally, in order to demonstrate

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the efficacy of the method, the performance of AIV-rNP-ELISA was compared with that of a commercial ELISA kit.

MATERIALS AND METHODS

Virus, RNA extraction and reverse transcription

An H4N6 avian influenza A virus isolate was propagated in specific pathogen-free (SPF) chicken embryonated eggs. The eggs were incubated at 37°C for 40 h; next, the allantoic fluid was collected, clarified by centrifugation, and stored at -70°C (Upadhyay et al., 2009). Viral RNA was extracted from allantoic fluid of inoculated eggs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The cDNAs were synthesized according to instructions provided with the superscript reverse transcriptase kit (Invitrogen). The reverse transcription reaction (RT) was performed using the superscript reverse transcriptase kit (Invitrogen) according to the manufacturer's recommendations with the extracted RNA as template. The complementary DNA (cDNA) obtained was used to amplify the NP by PCR.

Amplification of the AIV NP gene

Specific primers for the NP gene (forward + [5'-ATGCACATCATGGCGTCTCAA-3'] and reverse − [5'-TGATGGAGTCCATTGTTCCA-3']) described by Jin et al. (2004) and Yang et al. (2008) were designed with the modifications required for subsequent gene cloning in a pET-SUMO vector (Invitrogen) and used to amplify a 1128-bp region of the NP gene that codes from amino acid 1 to amino acid 376 and includes the major NP antigenic epitopes. The following cycling conditions were used for PCR: 35 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2.5 min, followed by a final extension step at 72°C for 10 min. The PCR product was analyzed by 1% agarose gel electrophoresis.

Cloning of NP-AIV gene and recombinant NP expression (rNP)

The purified PCR product of NP gene was inserted into a pET SUMO vector (Invitrogen) following the manufacturer's indications after cloning in TOP10F' *E. coli* competent cells (Invitrogen). This plasmid construction was used to transform competent BL21 *E. coli* cells, from which the plasmids were analyzed after purification, by nucleotide sequencing to confirm the presence of the gene insert. The *E. coli* BL21 cells transformed with the recombinant pET SUMO vector containing the NP gene were grown in Luria Bertani medium and protein expression was induced using 1.0 mM isopropyl-β-d-thiogalactopyranoside (IPTG) at 37°C for 16 h. The cell pellets were retrieved after centrifugation of *E. coli* culture at 12,000 × *g* for 10 min, diluted in lysis buffer (50 mM K_2HPO_4/KH_2PO_4, 400 mM NaCl, 100 mM KCL, 10% glycerol, 0.5% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, and 10 mM imidazole pH 7.8), and sonicated. The soluble fraction of this culture containing expressed rNP was separated after centrifugation at 12,000 × *g* for 10 min and purified in nickel-agarose resin (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's recommendations. Fractions (1-ml) were collected from the column and their protein concentrations were determined by the Bradford method (Bradford, 1976). Non-purified and purified preparations of recombinant NP were characterized by SDS-PAGE and western blot according to protocol followed by Mahmood and Yang (2012).

In silico analysis of recombinant AIV-NP antigenicity and epitope prediction

The deduced amino acid sequence from the rNP nucleotide sequence cloned in this study was analyzed for potential epitopes and compared with other deduced AIV-NP amino acid sequences deposited in GenBank, including GU052384 Czechoslovakia/1956 (H4N6), M22579 A/swine/Germany/2/1981 (H1N1), CY067273 A/mallard/Postdam/178-CIP046-qa6rnd2/1983 (H2N2), CY005555 A/duck/Hong Kong/7/1975 (H3N2), CY092164 A/duck/Western Australia/8108/1984 (H4N6), CY005570 A/duck/Hong Kong/365/1978 (H4N6), CY015084 A/chicken/Scotland/1959 (H5N1), CY005614 A/duck/Hong Kong/d134/1977 (H6N2), CY130153 A/turkey/England/1963 (H7N3), and CY005634 A/duck/HK/784/1979 (H9N2). The Hopp and Woods method (Hopp and Woods, 1981) included in BioEdit Sequence Alignment Editor Version 7.0.2 was used to evaluate the hydrophilicity profiles of rNP amino acid sequences expressed in this study and other NP amino acid sequences from other AIV subtypes. Recombinant NP epitope prediction was performed using the Bepipred Linear Epitope Prediction method (Larsen et al., 2006) (http://tools.immuneepitope.org/bcell/).

Chicken serum samples

A pool of 10 serum samples from specific-pathogen free (SPF) chickens was used as negative sera. The positive serum samples were provided by the National Veterinary Services Laboratories of the United States Department of Agriculture (NVSL, USDA). An additional set of 121 chicken serum samples was provided by the National Laboratory for Agriculture, São Paulo (LANAGRO/SP), which is the national reference laboratory for poultry diseases in Brazil.

Indirect ELISA (AIV-rNP-ELISA)

AIV-rNP-ELISA was performed according to the general protocol developed by Silva et al. (2014). A checkerboard titration of four rNP concentrations (2, 4, 8, and 16 µg/ml) and six dilutions (1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600) of the positive and negative serum controls was performed to determine the optimum antigen concentration and the ideal serum dilution. The microplate wells were coated with 50 µl of the purified recombinant antigen diluted in carbonate-bicarbonate buffer (0.05M, pH 9.6) and the reaction was incubated for 16 h at 4°C. Next, the microplates were washed four times with PBS (pH 7.4) + 0.05% Tween 20 (PBST) and the non-specific binding sites were blocked with 10% skim milk powder in PBST (100 µl per well) (blocking buffer) followed by incubation for 45 min at 37°C. Following another washing cycle, the chicken serum samples were diluted in blocking buffer, added to the microplates (50 µl per well), and incubated for 1 h at 37°C. After this step, the microplates were washed and treated with 50 µl per well of rabbit anti-chicken IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MI, USA) diluted 1:1000 in blocking buffer for 1 h at 37°C. The substrate-chromogen solution (0.05 M citrate-phosphate buffer pH 5.0; 0.04% σ-phenylenediamine and freshly added 0.006% H_2O_2) was added and the colorimetric reaction was stopped by adding 2M HCL after 15 min, and the optical densities (ODs) were determined at 490 nm using ELISA reader (Bio-Rad, Hercules, CA, USA). For each test serum sample, the mean OD (ODMTS) was expressed in relation to the positive reference serum mean OD (ODMPRS) and the negative reference serum mean OD (ODMNRS), as a sample to positive ratio (S/P), according to the formula S/P = ODMTS − ODMNRS/ODMPRS − ODMNRS. The cutoff point was the mean S/P value + 3 standard deviations calculated from 10 AIV-negative chicken serum samples (Gibertoni

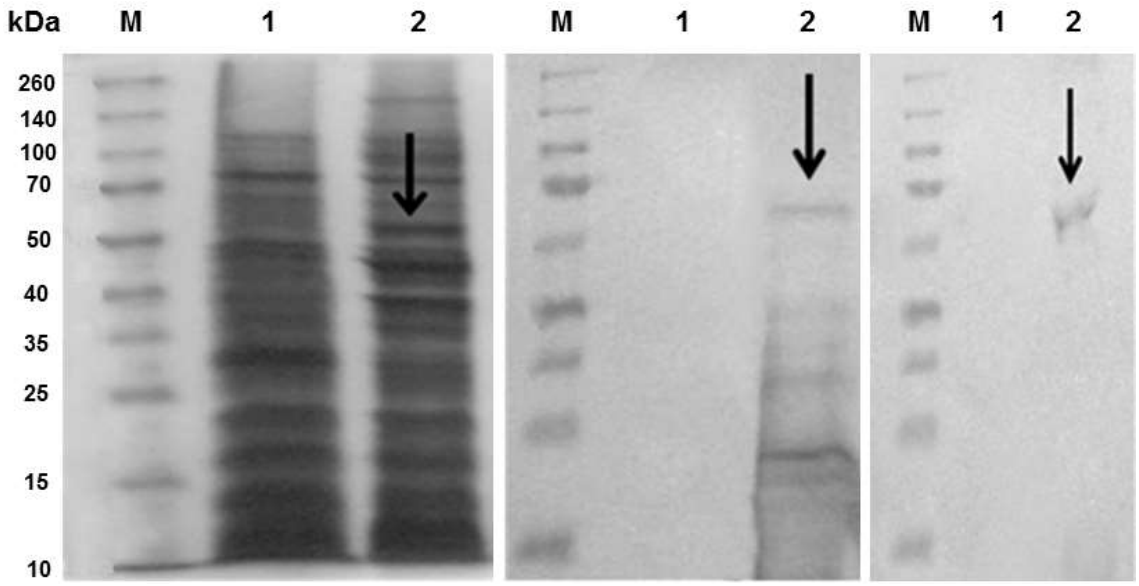

Figure 1. SDS-PAGE and western blot analysis of the AIV rNP expressed in *E. coli*. (A) SDS-PAGE. Column M: molecular weight marker; lane 1: non-induced fraction of *E. coli* culture (negative expression control); lane 2: *E. coli* induced fraction containing the rNP expressed. (B) Western blot of crude extract of rNP expressed in *E. coli* probed with anti-His monoclonal antibody. Lanes M, 1, and 2 received the same reagents as described for (A) except the samples were not purified. (C) Western blot of affinity-purified rNP fraction; lane M: molecular weight marker; lane 1: non-induced fraction of *E. coli* culture after chromatography in nickel-agarose resin; and lane 2: purified fraction of rNP after chromatography in nickel-agarose resin, probed with anti-His antibody. Black arrows indicate rNP.

et al., 2005).

Commercial indirect ELISA for the detection of anti-AIV antibodies

The IDEXX Avian Influenza Antibody Test kit (IDEXX Laboratories, Westbrook, ME, USA) was used according to the manufacturer's instructions to test the set of 121 chicken serum samples. The results were compared with those of rNP-ELISA.

Statistical analysis

The rNP-ELISA results were compared with results from a commercial indirect ELISA (AI Ab Test kit, IDEXX Laboratories) that uses whole virus as antigen. Sensitivity, specificity, accuracy, and agreement (κappa coefficient) values for rNP-ELISA were determined in comparison to the IDEXX kit according to Mohan et al. (2006). For the kappa coefficient (k), $k < 0.2$ indicates low agreement, $0.2 < k < 0.4$ indicates weak agreement, $0.4 < k < 0.6$ indicates moderate agreement, $0.6 < k < 0.8$ indicates good agreement, and $k > 0.8$ indicates a high level of concordance between tests (Landis and Koch, 1977).

RESULTS

Construction and expression of rNP in *E. coli*

A 1128-bp fragment was amplified and cloned from an H4N6 AIV isolate, encoding NP amino acids 1 to 376, into a pET SUMO vector. Nucleotide sequencing of the recombinant vector confirmed the presence and correct insertion of the NP gene, which had 99.72% identity with the nucleotide sequence deposited in GenBank (GU052384 of A/duck/Czechoslovakia/1956 [H4N6]) – a single amino acid change (T373A) in the deduced amino acid sequences. It was also confirmed that the NP gene fragment was cloned in frame with the SUMO peptide and poly-histidine tag genes. The expressed NP was a soluble recombinant protein with a molecular weight (MW) of approximately 56 kDa, by SDS-PAGE and western blot analysis (Figure 1). Line 2 of Figure 1B shows bands corresponding to SUMO peptide plus the poly-histidine tag (~13 kDa) and other proteins from *E. coli* due to the fact that this sample was not purified. However, after purification in nickel-agarose resin, only the rNP band was detected in the western blot (Figure 1C, line 2). The MW observed (~56 kDa) corresponds to a NP fragment (~43 kDa) fused to the SUMO peptide and poly-histidine tag (~13 kDa). A 1 mg/ml yield of rNP was obtained from 1000 ml of transformed BL21 *E. coli* culture, after purification of the protein on nickel-agarose resin.

In silico analysis of rNP antigenicity

The Bepipred Linear Epitope Prediction method

Table 1. Amino acid residues and respective positions and sizes for the 15 AIV recombinant NP epitopes predicted by Bepipred Linear Epitope Prediction.

Epitope	AA start	AA end	Peptide	Size
1	1	25	MASQGTKRSYEQMETGGERQNATEI	25
2	73	74	ER	2
3	76	99	NKYLEEHPSAGKDPKKTGGPIYRR	24
4	102	103	GK	2
5	123	131	ANNGEDATA	9
6	144	149	NDATYQ	6
7	159	160	MD	2
8	171	184	TLPRRSGAAGAAVK	14
9	206	214	FWRGENGRR	9
10	231	233	QTA	3
11	243	252	RESRNPGNAE	10
12	289	293	YDFER	5
13	318	325	PNENPAHK	8
14	352	360	VVPRGQLST	9
15	364	374	QIASNENMEAM	11
AA: amino acid		-	-	-

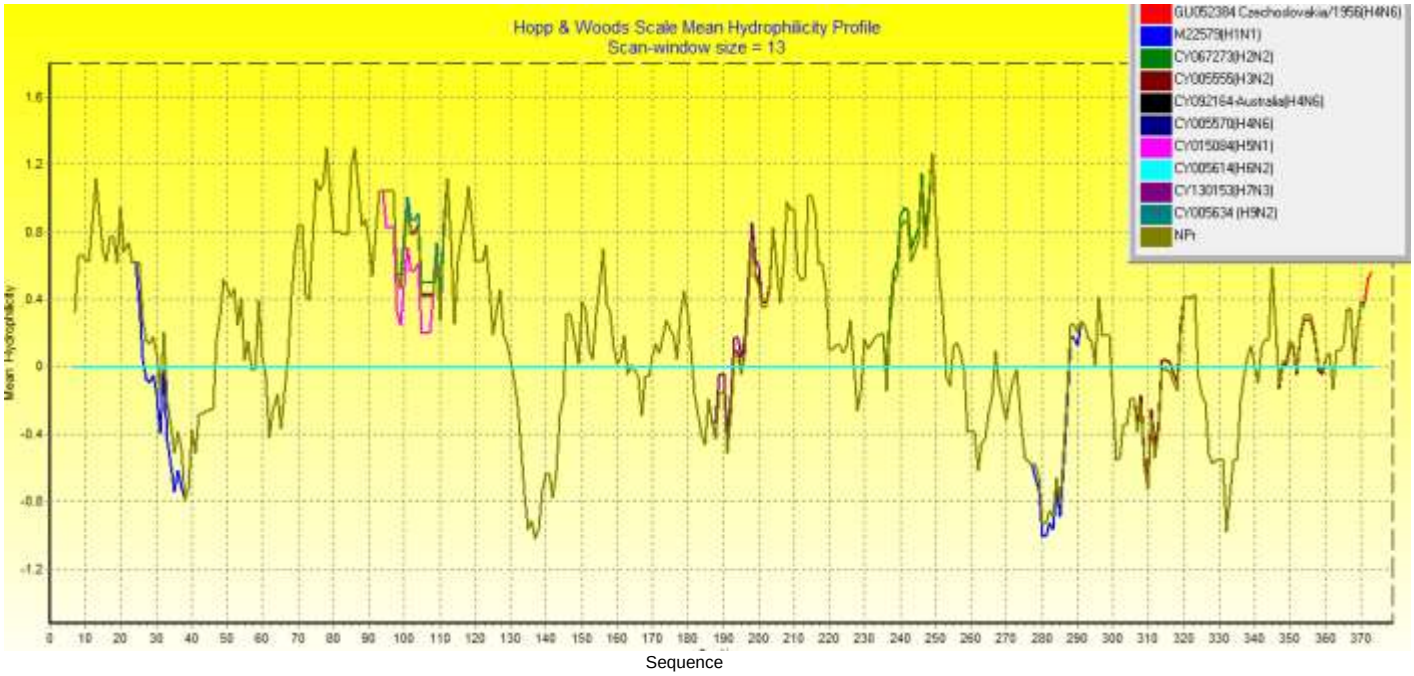

Figure 2. Comparison of hydrophilicity profiles by the Hopp and Woods method between amino acid sequences from AIV recombinant NP predicted epitopes and deduced amino acid sequences from AIV NP genes deposited in GenBank. Each amino acid sequence is represented by one color and identified in the plot legend.

determined fifteen epitopes from the deduced amino acid sequence of rNP (Table 1). To confirm this, the Hopp and Woods method was used (Hopp and Woods, 1981) to generate a hydrophilicity profile analysis by aligning the rNP deduced amino acid sequences in this study with amino acid sequences deduced from the NP gene from other AIV strains deposited in GenBank (Figure 2). This analysis predicted nearly the same 15 potential epitopes of these proteins, corresponding to the oligopeptides encompassing the amino acid residues in the

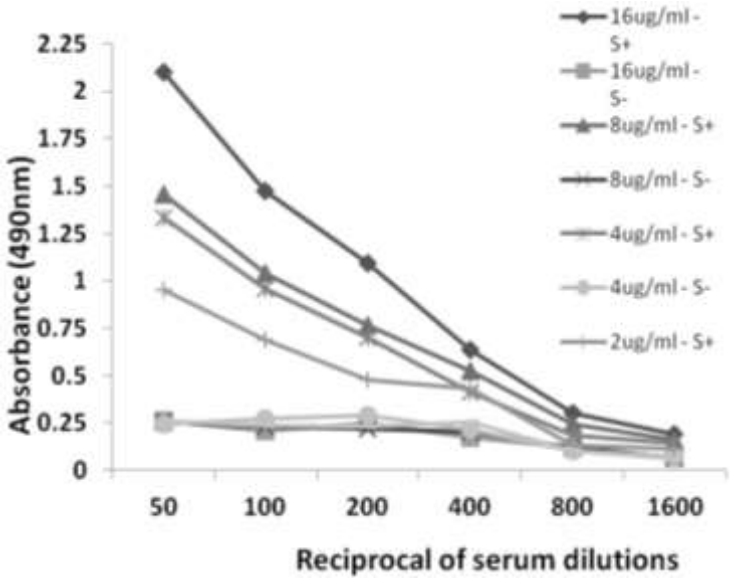

Figure 3. Checkerboard titration results of indirect ELISA for different concentrations of the rNP antigen against different dilutions of AIV-positive (S+) and AIV-negative (S-) reference chicken sera.

hydrophilicity peaks above the threshold value (green line = 0). The great similarity between the hydrophilicity peaks of the rNP amino acid sequences and those of the other NP sequences analyzed indicated that most potential rNP epitopes are conserved in NP sequences from different AIV subtypes (Figure 2).

Indirect AIV-rNP-ELISA

The checkerboard titration showed that, in indirect AIV-rNP-ELISA, AIV-positive serum reacted in a dose-dependent manner as rNP concentration increased from 2 to 16 µg/ml and, conversely, declined as the AIV-positive serum was diluted (Figure 3). An rNP concentration of 8 µg/ml and a single serum dilution of 1:100 were selected as ideal for the analysis of chicken test sera in AIV-rNP-ELISA (Figure 3) because, under these conditions, the highest specific reactivity between the chicken antiserum against AIV was found and difference was observed in the optical density values obtained from AIV-positive and AIV-negative serum controls. Analysis of 10 AIV-negative serum samples defined a cutoff S/P value of 0.125 for rNP-ELISA.

Comparative analysis between AIV-rNP-ELISA and commercial ELISA (AI Ab Test, IDEXX)

Table 2 compares the performance of rNP-ELISA in detecting anti-AIV antibodies with that of IDEXX ELISA (IDEXX Laboratories), in a set of 121 chicken serum samples. The sensitivity, specificity, and accuracy of rNP-ELISA were 95.0, 97.0, and 96.7%, respectively. The agreement of κ = 0.88 indicated a high similarity in the performance of the two ELISA tests.

Table 2. Comparison of AIV-rNP-ELISA and IDEXX AI Ab test for the detection of anti-AIV antibodies.

AIV-rNP-ELISA	IDEXX AI Ab Test		
	Positive	Negative	Total
Positive	19	3	22
Negative	1	98	99
Total	20	101	121

Sensitivity = 95.0%; specificity = 97.0%; accuracy= 96.7%; agreement (kappa index) = 0.88.

DISCUSSION

The pET SUMO vector-*E. coli* cloning and expression system used in this study resulted in the expression of a relevant quantity of a soluble form of AIV rNP that conserved most of the antigenicity of the original viral NP. One of the reasons for this result may be the effect of the SUMO peptide fusion in enhancing the solubility of viral recombinant proteins expressed in *E. coli* (Guerrero et al., 2015; Shafer et al., 1998; Zuo et al., 2005)

Recombinant NP epitope prediction confirmed that most antigenic sites of this recombinant protein were conserved compared to the NP of homologous (H4N6)

and heterologous (H1N1, H2N2, H3N2, H5N1, H6N7, and H9N2) AIV subtypes. In addition, some amino acid residue stretches predicted as rNP epitopes in this study, such as 71 to 96 and 290 to 353, have been previously confirmed as target epitopes of monoclonal antibodies to NP from AIV subtype H1N1 (Varich and Kaverin, 2004; Yang et al., 2008). These findings are further supported by the reactivity of rNP to chicken AIV-specific antibodies in the indirect ELISA developed in this study.

The functionality of AIV-rNP-ELISA was evaluated by analysis of 121 field chicken serum samples. Results showed high sensitivity (95%), specificity (97%), accuracy (96.7%), and good agreement (k = 0.88). compared to a commercial ELISA kit. Although, there are no optimum definitive values of sensitivity, specificity and agreement of a serological test (OIE, 2015), the sensitivity, specificity and agreement values of the current study are in the range from those found for these parameters in the analysis of commercial ELISAs and other indirect ELISA methods using recombinant NP preparations from different AIV strains. Upadhyay et al. (2009) found 98% sensitivity and 97% specificity compared to the commercially available ProFLOK® AIV Plus ELISA kit (Synbiotics, Kansas City, MO, USA). However, the concentration of yeast-expressed recombinant NP used to coat the ELISA microplate wells (80 μg/ml) was ten-fold higher than in the present study (8 μg/ml). Similarly, Wu et al. (2007), comparing the results of an indirect ELISA developed with an insoluble fraction of a recombinant NP expressed in another *E. coli* system with HI, AGID, and commercial ELISA kit IDEXX FlockChek™ (IDEXX Laboratories), reported a concordance of 92.0, 83.3, and 96.2%, respectively, but these authors used a higher concentration (50 μg/ml) of recombinant NP.

Overall, our findings indicate that the cloning and expression of a partial NP fragment in a pET SUMO-*E. coli* system yielded a soluble form of a recombinant viral antigen containing the most important epitopes of the protein. This approach contributed to the development and application of rNP-ELISA, with a performance similar to that of a commercial ELISA kit. Thus, the ELISA developed here using a soluble form of rNP offers a viable alternative to improve the immunodiagnosis of AIV infection in chickens, as it was able to analyze rapidly, simply and inexpensively a large number of serum samples with a low concentration of rNP compared with others studies and commercials kits. In addition, the rNP-ELISA has the potential to detect chicken antibodies against different AIV subtypes, because this technique uses a highly conserved antigen (NP) among the different subtypes of AIV, as demonstrated the epitope prediction analysis of this study and the results from previous studies reported by (Jin et al., 2004; Upadhyay et al., 2009; Wu et al., 2007). Despite the current rNP-ELISA was not tested for the detection of anti-AIV antibodies of other avian species, the rNP can be used also as coat-antigen in other ELISA methods such as blocking-ELISAs to react with anti-NP monoclonal antibodies (Jensen et al., 2013) to replace the purified AIV particles obtained from AIV-infected SPF embryonated chicken eggs that are submitted to purification by ultra-centrifugation in sucrose gradient.

The diagnosis of AI is achieved either by direct methods such as viral isolation, detection of genomic RNA, and viral proteins in biological samples, or by indirect methods involving the detection of AIV-specific antibodies (OIE, 2015) as performed in this study. The different diagnostic procedures available to diagnosis AI have to be able to handle a large number of samples and generate prompt and accurate results. Thus, the availability of appropriate reagents is of great importance, especially when serological tests are used for this purpose. Nowadays, most of the commercial kits for the detection and monitoring of chicken AIV-specific antibodies with ELISA use antigenic preparations from purified viral particles obtained by ultra-centrifugation, a very time-consuming and expensive method. Molecular cloning and expression of viral recombinant proteins, as described here, can provide a more refined and accessible antigen preparation such as AIV recombinant NP for use in serological techniques for the diagnosis of infection caused by this virus.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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Antimicrobial susceptibility patterns of *Staphylococcus aureus* and coagulase negative staphylococci isolated from humans in Nairobi, Kenya

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Nosocomial infections due to multidrug resistant *Staphylococcus aureus* are an important health problem worldwide. Antimicrobial resistance prolongs the duration of hospitalization, thereby increasing the cost of patient care. For a long time, methicillin was considered as drug of choice for treatment of penicillin-resistant staphylococcal infections. Emergence of methicillin resistance reduced the available options for treatment of nosocomial and community acquired *S. aureus*. Normally, such strains are only sensitive to glycopeptides such as vancomycin and teicoplanin. Recent reports show that methicillin resistant *S. aureus* (MRSA) have become multiply resistant to other drugs such as fluoroquinolones, trimethoprim-sulfamethoxazole (SXT), clindamycin or erythromicin and there are reports of vancomycin resistant strains from different parts of the world. The aim of this study was to determine the susceptibility patterns of *Staphylococcus* isolates from humans. Drug susceptibility testing of isolates was determined using the disk diffusion method. A total of 110 *S. aureus* (SA) and 23 coagulase negative staphylococcus (CoNS) isolates from human sources were studied. Both SA and CoNS isolates were completely sensitive to vancomycin. On one hand, there was a comparable high resistance for both SA and CoNS to penicillin G, augmentin and tetracycline. On the other hand, there was significantly high resistance to erythromycin (69.6%), SXT (69.6%), oxacillin (82.6%), ciprofloxacin (52.2%) and clindamycin (39.1%) among CoNS when compared with SA isolates (erythromycin 38.2%, SXT 38.2, oxacillin (33.6%), ciprofloxacin (26.4%), clindamycin 18.2%) with p values 0.0090, 0.0099, 0.0001, 0.0239 and 0.0483, respectively. These high levels of resistance, calls for continuous surveillance studies to monitor for *S. aureus* infections in the community and hospital settings and the emergence of vancomycin resistant isolates.

Key words: Methicillin resistant, *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* (MRSA), antibiotic susceptibility, vancomycin, coagulase negative staphylococci.

INTRODUCTION

Staphylococcus aureus is a common cause of both community and hospital-acquired infections. Clinical

syndromes associated with severe disease include bacteraemia, pneumonia, endocarditis, septic arthritis, osteomyelitis and deep abscess formation (Enright et al., 2000; Loir et al., 2003, Abdulgader et al., 2015).

Mortality from invasive *S. aureus* disease was high during the pre-antibiotic era. However, the introduction of penicillin in the 1930s had a dramatic impact on the treatment of *S. aureus* infections. The semisynthetic penicillin methicillin was introduced in 1959 to overcome the problems that arose from the increasing prevalence of penicillinase-producing isolates of *S. aureus* resistant to penicillin G and penicillin V. However, methicillin-resistant *S. aureus* (MRSA) strains rapidly emerged and became a major clinical problem within hospitals during the 1960s in Europe and the 1970s in the United States and elsewhere (Enright et al., 2000). MRSA was first reported in England in 1961, shortly after its introduction (Mulvey et al., 2001). Worldwide reports show that MRSA strains are resistant to most other classes of antimicrobial agents and are susceptible only to glycopeptides and a few new investigational drugs (Enright et al., 2000; Lee, 2003).

MRSA is common also in the African region (Falagas et al., 2013). From an African multicentre study by Kesah et al. (2003), methicillin resistance was detected in 213 (15%) of the 1440 isolates tested. In another study, the rate of MRSA was relatively high in Nigeria, Kenya and Cameroon (21 to 30%) and below 10% in Tunisia, Malta and Algeria. More than 60% of MRSA were multidrug resistant, with relatively high resistance to erythromycin, gentamicin and oxacillin. Fusidic acid, co-trimoxazole, rifampicin and ciprofloxacin exhibited moderate efficacy. All MRSA isolates were sensitive to vancomycin (Kesah et al., 2003). In a 1995 study conducted in Nairobi, it was found that about 90% of patients admitted in burn units were infected with MRSA and thereby significantly increasing duration of stay in hospital and treatment cost (Muthotho et al., 1995).

The determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infected patients. This requirement is crucial especially at a time when reports indicate that resistance is increasing and there is emergence of multidrug resistant microorganisms. Standard procedures and breakpoints have been defined to predict therapeutic outcome both in time and at different geographic locations (Fluit et al., 2001).

Monitoring of multidrug resistant methicillin resistant *S. aureus* is therefore an important public health aspect in Kenya, where MRSA may increase the duration of stay in hospital and treatment costs. In addition, drugs used to treat MRSA are not readily available and affordable in this country.

The aim of this study was to determine the proportion of MRSA and investigate the antimicrobial susceptibility patterns of both coagulase positive and coagulase negative staphylococcal strains isolated from humans in Nairobi, to commonly used antibiotics and vancomycin.

MATERIALS AND METHODS

This was a descriptive and cross sectional study of staphylococci isolates obtained from studies on wounds and blood borne infections and stored at the Centre for Microbiology Research-Kenya Medical Research Institute.

Identification of *Staphylococcus* species

Samples were subcultured aerobically and growing colonies were Gram stained. Gram-positive cocci clusters were tested for production of catalase and coagulase enzymes and confirmed by API Staph Identification system (BioMerieux Inc., Durham, USA). Confirmed isolates were stored in Tryptic soy broth containing 15% glycerol and frozen at -80°C until further processing.

β-Haemolysis test

Isolates confirmed as *S. aureus* were inoculated onto blood agar and incubated for 18 to 24 h. Isolated colonies were picked and re-suspended in sterile normal saline (0.85%) to give a final inoculums equivalent 0.5 McFarland turbidity standard before inoculating them on Mueller Hinton agar (Oxoid, Hampshire, UK) plates each containing 20 ml of the medium to attain a uniform depth of 4 mm.

Antimicrobial susceptibility testing

All isolates were tested for susceptibility to a panel of 12 antibiotics (penicillin 10 units, ciprofloxacin 5 µg, chloramphenicol 30 µg, clindamycin 2 µg, gentamicin 10 µg, erythromycin 15 µg, oxacillin 1 µg, amoxycillin/clavulanic acid (Augmentin) 10/20 µg, tetracycline 30 µg, sulphamethoxazole-trimethoprim 25 µg, methicillin 5 µg and vancomycin 30 µg) by using the disk diffusion technique following guidelines of Clinical and Laboratory Standards Institute (CLSI, 2002).

The antibiotic disks were placed on each agar plate at equal distance from each other and plates were incubated aerobically at 35°C for 16 - 18 h, except for vancomycin, methicillin and oxacillin which were incubated for 24 h. *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 35218 were used to control for growth of bacteria and efficacy of antibiotic disks. The size of zones of inhibition were recorded and interpreted according to CLSI standards (CLSI, 2015).

RESULTS

Identification of *Staphylococcus* species

A total of 133 staphylococcal isolates were studied. Out

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Table 1. β Haemolysis patterns of the staphylococci isolates.

Isolate	Beta (β) haemolytic N (%)	Non-haemolytic N (%)	Total
Staphylococcus aureus	98 (89.1)	12 (10.9)	110
CoNS	19 (82.7)	4 (17.3)	23
Total	117 (88.0)	16 (12.0)	133

CoNS = Coagulase-negative staphylococci.

Table 2. Antimicrobial susceptibility of 133 *Staphylococcus* species to 12 antibiotics.

Antibiotic	Resistant N (%)	Intermediate N (%)	Sensitive N (%)
Penicillin	122(91.7)	0(0.0)	11(8.3)
Augmentin	100(75.2)	0(0.0)	33(24.8)
Tetracycline	62(46.6)	2(1.5)	69(51.9)
Erythromycin	58(43.6)	13(9.8)	62(46.6)
SXT	58(43.6)	9(6.8)	66(49.6)
Oxacillin	56(42.1)	0(0.0)	77(57.9)
Methicillin	46(34.6)	7(5.3)	80(60.2)
Ciprofloxacin	41(30.8)	4(3.0)	88(66.2)
Chloramphenicol	40(30.1)	5(3.8)	88(66.2)
Gentamicin	38(28.6)	2(1.5)	93(69.3)
Clindamycin	29(21.8)	9(6.8)	95(71.4)
Vancomycin	0(0.0)	0(0.0)	133(100.0)

SXT= Sulfamethoxazole-Trimethoprim.

of these, 110 (82.7%) were *S. aureus* (SA) and 23 (17.3%) coagulase-negative staphylococci (CoNS).

β-Haemolysis in *Staphylococcus* species studied

In total, 88% of all isolates were β haemolytic and the rest were non hemolytic. *S aureus* isolates were significantly more β hemolytic in comparison with the CoNS isolates (Table 1).

Antimicrobial susceptibility of Staphylococcus species

All isolates were highly sensitive to vancomycin (100%) but highly resistant to penicillin G (91.7%). The order of increasing resistance was penicillin G > augmentin > tetracycline > erythromycin = SXT > oxacillin > methicillin > ciprofloxacin > chloramphenicol > gentamicin > clindamycin > vancomycin (Table 2). Isolates were relatively more sensitive to gentamicin and clindamycin showing resistance in only less than 30% of all isolates.

Comparison of antibiotic susceptibility of *S. aureus* and coagulase negative *Staphylococcus* species

Both *S. aureus* and coagulase-negative staphylococci isolates were completely sensitive to vancomycin. As indicated in Table 3, *S. aureus* were more resistant to most drugs except oxacillin, when compared with coagulase-negative staphylococci (Table 3). There was significantly high resistance among coagulase-negative staphylococci isolates from human sources to erythromycin (69.6%), SXT (69.6%), oxacillin (86.2%), ciprofloxacin (52.2%) and clindamycin (39.1%) as compared to *S. aureus* isolates from human sources (erythromycin 38.2%, SXT 38.2, clindamycin 18.2%) with P values 0.0090, 0.0099, 0.0001, 0.0239 and 0.0483, respectively) (Table 5).

There was significantly higher resistance to oxacillin in coagulase-negative staphylococci than in *S. aureus.* The percentage resistance of *S. aureus* and coagulase-negative staphylococci to oxacillin was 29.2 and 55.7% (P value = 0.0003), respectively. Overall resistance to penicillin and augmentin was significantly higher for *S. aureus* than coagulase-negative staphylococci (P values = 0.0001 for both).

Table 3. Comparison of overall resistance of *S. aureus* and coagulase- negative staphylococci.

Antibiotic	*Staphylococcus aureus* resistant N (%)	CoNS resistant N (%)	P value[a]
Penicillin	101(91.8)	21(91.3)	1.0000
Augmentin	82(74.5)	18(78.3)	0.7967
Tetracycline	50(45.5)	12(52.2)	0.6479
Erythromycin	42(38.2)	16(69.6)	0.0099
SXT	42(38.2)	16(69.6)	0.0099
Oxacillin	37(33.6)	19(82.6)	0.0001
Methicillin	35(31.8)	11(47.8)	0.1550
Ciprofloxacin	29(26.4)	12(52.2)	0.0239
Chloramphenicol	27(24.5)	13(56.5)	0.0768
Gentamicin	30(27.3)	8(34.8)	0.4583
Clindamycin	20(18.2)	9(39.1)	0.0481
Vancomycin	0(0.0)	0(0.0)	-
Total	110(100)	23(100)	

[a]Fisher's exact test; SXT = sulfamethoxazole-trimethoprim; CoNS=coagulase-negative staphylococci.

Table 4. Proportion of isolates resistant to one or more of the eleven antibiotics tested.

Isolate	Total isolates	Resistant to Number of antibiotics											
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Staphylococcus aureus	110	8	13	25	15	7	5	3	5	5	7	9	8
CoNS	23	1	0	2	1	3	1	2	1	4	3	2	3
Total (%)	133 (100)	9 (6.8)	13 (9.7)	27 (20.3)	16 (12.0)	10 (7.5)	6 (4.5)	5 (3.8)	6 (4.5)	9 (6.8)	10 (7.5)	11 (8.3)	11 (8.3)

CoNS =Coagulase-negative staphylococci.

Table 5. Summary of multidrug resistance for all *S. aureus* and CoNS isolates.

Isolate	Sensitive N (%)	Resistant to 1 or 2 N (%)	MDR N (%)	Total no. of isolates
Staphylococcus aureus	8 (7)	38 (35)	64 (58)	110
CoNS	1 (4)	2 (9)	20 (87)	23
Overall total	9 (6.8)	40 (30.0)	84 (63.2)	133

[a]Fisher's exact test; CoNS =coagulase-negative staphylococci.

MRSA were detected by determining the susceptibility to oxacillin or methicillin. As shown in Table 3, SA isolates were more resistant to oxacillin than methicillin (33.6 and 31.8% respectively). In contrast, CoNS showed a different trend with oxacillin and methicillin isolates at the proportion of 82.6 and 47.8%, respectively. Out of 12 drugs that were tested, only vancomycin completely inhibited the growth of all isolates tested.

Only 7% of all isolates were completely sensitive to all antibiotics (Tables 4 and 5). Sixty three percent of all isolates were multidrug resistant (those resistant to three or more drugs). The remaining 30% of the isolates were resistant to one or two drugs (Table 5). Approximately 8% of all isolates were resistant to all the tested drugs except vancomycin. Other isolates were resistant to one or two drugs (9.7 and 20.3%, respectively). Isolates resistant to between three and ten drugs varied between 4.5 and 12% of all isolates (Table 5). Considered individually, the proportion of multidrug resistant (MDR) isolates in *S. aureus* and coagulase negative was 58 and 87%,

respectively.

DISCUSSION

This study shows that the commonly available drugs such as penicillin, augmentin, tetracycline, erythromycin and SXT are no longer reliable in treating nosocomial and community acquired staphylococcal infections. For instance, out of all isolates tested, 11 were resistant to all antibiotics except vancomycin. Some reports indicate increase of vancomycin intermediate/resistant *S. aureus* (VISA/VRSA) isolates worldwide (Lowy, 2003; Monaco et al., 2016). Vancomycin resistance became of more concern since the demonstration of successful transfer of the *vanA* gene from enterococci to *S. aureus* under laboratory conditions (Noble et al., 1992) and after reports of staphylococcal isolates resistant to vancomycin in some countries (Hiramatsu et al., 1997a, b; Sieradzki et al., 1999; Chang et al., 2003; Whitener et al., 2004; Palazzo et al., 2005; Lee et al., 2015).

Because isolates in this study did not show resistance to vancomycin, it is advisable to limit the use of glycopeptides to treat only MRSA infections so as to reduce the selective pressure and likely emergence of resistance to this class of antibiotics. Decreased staphylococcal susceptibility to vancomycin is not due to transfer of *vanR* genes from vancomycin-resistant enterococci (VRE) or to small colony variants, as noted in staphylococci for other antimicrobial agents (Mitsuyama et al., 1997) but appears to be a gradual selection process due to treatment pressure. Glycopeptide - resistant mutants of *S. aureus* have been experimentally selected by increasing the levels of vancomycin present during *in vitro* growth (Daum et al., 1992; Sieradzki and Tomasz, 1997). The use of avoparcin, another member of the glycopeptide class of antibiotics, as a growth-promoting agent in the production of food animals is often cited as playing a role in the spread of glycopeptide-resistant microorganisms (Aarestrup et al., 1996; Van den Bogaard et al., 1997; Witte, 1997, Economou and Gousia, 2015). Although, it is well recognized that vancomycin resistance is more prevalent in the United States than in Europe, it has not been explained why avoparcin usage fails to correlate with the different epidemiologies of resistance between the two continents; avoparcin was never approved for use in animals in the United States, in contrast to its broad use as a growth-promoting agent in Europe (Donnelly et al., 1996; Leclercq and Courvalin, 1997). There is no explanation yet, to this continental variation and more theories are required to explain the difference in the glycopeptide resistance. Based on the outcome of this study and available literature, the use of avoparcin should also be controlled to avoid spread of resistance. In order to avoid the emergence of glycopeptide resistance in currently susceptible staphylococci isolates, the use of avoparcin in Kenya should also be highly controlled. The fact that the use of glycopeptides should be controlled is supported by a number of reports. According to a study by Tenover et al. (1998) glycopeptide-intermediate *S. aureus* (GISA) isolates represented mutants selected *in vivo* with increased resistance to glycopeptides as a result of prolonged exposure of the organisms to constant levels of vancomycin in an opportune environment.

In the current study, all isolates had vancomycin zones greater than 14 mm. According to CLSI guidelines, any staphylococcal isolates with zones diameters of 14 or less should be tested by an MIC method. When it is determined that isolates have elevated MIC to vancomycin (>= 4µg/ml), these should be sent to a reference laboratory (CLSI, 2015). According to a study by Tenover and et al. (1998), the disk diffusion testing, which is widely used around the world does not differentiate strains with reduced susceptibility to vancomycin from susceptible strains (MIC range, 0.5 to 2 µg/ml). Accordingly, the disk diffusion test should not be used alone for testing staphylococci resistance with vancomycin. The same study suggests that disk diffusion tests with another glycopeptide, teicoplanin may be of value for identifying isolates with reduced susceptibility to glycopeptides. They also recommend the use of vancomycin agar screening test as method for testing staphylococci MICs and detect isolates with reduced glycopeptide susceptibility (Tenover et al., 1998).

The isolates showed a decreasing trend of resistance in the order, penicillin G > augmentin > tetracycline > erythromycin > SXT > oxacillin > methicillin > ciprofloxacin > chloramphenicol> gentamicin > clindamycin > vancomycin. Similar trends have been observed in South Africa (Marais et al., 2009). This indicates that the antibiotic class of penicillins has higher chances of treatment failure and should not be recommended for treatment of staphylococcal infections. Ciprofloxacin, chloramphenicol, gentamicin and clindamycin should be sought as alternative treatment for MRSA infections. Vancomycin is the most effective drug of all the tested antibiotics when β lactams are ineffective (Shakibaie et al., 2002; Casey et al., 2007; Gad et al., 2010). However, due to poor tissue diffusion and moderate bactericidal activity, vancomycin can be combined with rifampicin for deep-seated infections (Aubry-Damon et al., 1998).

According to the overall proportions of resistant isolates in this study, the authors arbitrary classified the antibiotics with respect to these isolates as highly resistant if percentage resistance is between 50 and 100% as was the case with penicillin and augmentin. Tetracycline, erythromycin, SXT, oxacillin, methicillin, ciprofloxacin, chloramphenicol and gentamicin were moderately resistant (resistance between 25 and 49%). Clindamycin was the only drug to which less than 25% of the isolates were resistant. Interestingly, all isolates were 100% susceptible to the antibiotic vancomycin.

A notable phenomenon with coagulase-negative

staphylococci is that they are significantly more resistant to most antibiotics and particularly to oxacillin as compared to *S. aureus*. This is consistent with previous studies by Reynolds et al. (2004) where 76% of coagulase-negative staphylococci isolates were oxacillin resistant when compared with 42% of *S. aureus*. In another study, rates of oxacillin resistance among *S. aureus* and CoNS isolates were 59.3 and 78.5%, respectively (Johnson et al., 2003). The *mec* gene responsible for *S. aureus* resistance is postulated to have originated from a different species of staphylococci. Although, many methicillin-resistant strains appear to be descendants of a limited number of clones, some appear to be multi-clonal in origin, suggesting the horizontal transfer of *mec* DNA (Archer and Niemeyer, 1994; Abdulgader et al., 2015). Hence it is possible that the higher resistance to oxacillin in these coagulase negative isolates could be a source to transfer *mec* gene to other staphylococci isolates. This was previously confirmed in studies by Wielders et al. (2002).

MRSA isolates often are multiply resistant to commonly used antimicrobial agents, including erythromycin, clindamycin and tetracycline, a situation that was evident in this study. MRSA isolates in this study were not only resistant to β-lactam antibiotics but also to chloramphenicol, clindamycin, ciprofloxacin, erythromycin, gentamicin and sulphamethoxazole-trimethoprim.

Both *S. aureus* and coagulase-negative staphylococci isolates from humans were highly resistant to penicillin and augmentin. However, about 9% of these isolates were sensitive penicillin. Treatment with these drugs should only be prescribed after sensitivity testing to penicillin.

Indiscriminate use and sale of antimicrobials, sale of antibiotics without prescription, sale of under dose preparations, brand substitution and self-medication can enhance the development of drug resistance (Indalo, 1997; Shakibaie et al., 2002, Roess et al., 2013). Therefore, to control the spread of MRSA, it is advised that both antibiotic use regulation and contact preventions be strictly observed. Similarly, culture surveillance is an important control measure (Farr, 2004; Drees et al., 2016).

Future work is required to determine the clonal relationship among MRSA isolates from a wider area of study, which can be used as epidemiological reference tool during MRSA outbreaks in hospitals and also aid in management and control of these infections. Using molecular tools, the relatedness between *S. aureus* and coagulase negative staphylococci can possibly indicate the ease with which staphylococci can transfer resistance genes among different genus.

Conclusion

Although, the study isolates were multidrug resistant, there was no isolate which was vancomycin resistant. The fact that 11 (8.3%) of all isolates were resistant to 11 out of 12 tested antibiotics is an alarming situation. Vancomycin should therefore be controlled in hospitals to avoid quick emergence of resistance to this life saving drug. There is a relatively high proportion of oxacillin resistance isolates among coagulase negative staphylococci, these may be responsible for the transfer of *MecA* gene responsible for oxacillin resistance to the more pathogenic *S. aureus* strains. Therefore, medical institutions should regularly perform vancomycin agar screening to determine any emergence of glycopeptide and other antibiotics resistance among staphylococci isolates.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Phenotypic detection of extended spectrum beta-lactamase in multidrug-resistant *Escherichia coli* from clinical isolates in Niamey, Niger

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Extended spectrum beta lactamase (ESBL) producing *Enterobacteriaceae* is one of the main causes of antibiotic treatment failure in hospitals. The aim of this study was to evaluate the prevalence of ESBL produced by multidrug-resistant (MDR) *Escherichia coli* isolated from various clinical samples (urine, stool, pus, blood culture) in the "Hôpital National de Niamey" and the "Hôpital National Lamordé" of Niamey, Niger. Samples were processed using standard bacteriological methods. Isolates were identified by biochemical tests and confirmed on API 20 E system (Bio-Mérieux, France). Antibiotic susceptibility was determined using the disk diffusion method on Mueller-Hinton (MH) agar plates (Liofilchem, Italy). Producing of extended spectrum beta-lactamase was performed using simple double-disk synergy test (DDST) and double-disk synergy test using cloxacillin. A total of two hundred and seventeen (217) multidrug-resistant *E. coli* were isolated from various clinical samples. Among these isolates, 57 (26.3%) were extended spectrum beta-lactamase producers. From clinical sources, prevalence of ESBL producing *E. coli* was observed in urine samples (26.7%), stool samples (26.3%), pus samples (25%) and blood samples (25%). ESBL producing *E. coli* were observed in the age groups under 5 years (24.9%), 26 to 45 (38.1%) and over 65 years (50%). This study showed a notable prevalence of extended spectrum beta-lactamase *E. coli* isolated from various clinical samples in two hospitals of Niamey, suggesting the rational and judicious use of antibiotics by clinicians.

Key words: Extended spectrum beta lactamase (ESBL), multidrug-resistance, *Escherichia coli*, prevalence, Niamey, Niger.

INTRODUCTION

Escherichia coli is a commensal of the human gut and one of the most frequently isolated bacteria from clinical

specimens (Quinet et al., 2010). It plays an important role as a member of the gut microbiota; however, pathogenic strains also exist, including various diarrheagenic *E. coli* pathotypes and extraintestinal pathogenic *E. coli* that cause illness like bacteremia, bladder infections, meningitis or pus (Dias et al., 2009; Smith et al., 2010; Fratamico et al., 2016). The discovery of antibiotics has been a humanity relief because these remedies have significantly reduced the incidence of infectious diseases, especially in developing countries (Guessennd et al., 2008). *E. coli* is one of the most common clinical pathogens causing nosocomial infection. For a long time, the widespread use of antibiotics to treat *E. coli* infectious disease has rapidly increased the multidrug resistance (MDR) of *E. coli* (Trecarichi et al., 2012; Kanwar et al., 2013) especially with those strains producing ESBL (Bush, 2001; Woerther et al., 2013). The appearance of ESBL stated in the 1980s and widely distributed in the world (Knothe et al., 1983; Bradford, 2001) and conferred increased resistance to beta-lactams except carbapenems and cephamycins (Patterson, 2001; Masterton et al., 2003). ESBLs are plasmid mediated and the genes encoding these enzymes are easily transferable among different bacteria (Todar, 2012). Most of these plasmids not only contain DNA encoding ESBLs but also carry genes conferring resistance to several non-*β*-lactam antibiotics (Rankin and Svara, 2011). The presence of ESBL in clinical isolate has been documented as a very serious problem and a significant trait to: quick survival of patients in the hospital, high economic burden, loss of hours in life's activities and high treatment failure (CDC, 2010). The phenotypic methods are currently the gold standard in determination of susceptibility or resistance of clinical isolates. The most widely used methods to screen ESBL are E-test, or double-disk synergy test (DDST) (EUCAST, 2014). Several reports have described the prevalence of ESBLs in the Middle East North Africa region and most of the Gulf Cooperation Countries (Zowawi et al., 2013). However, there is insufficient scientific data on the prevalence of ESBLs available from the State of Niger.

This study aimed to determine the prevalence of ESBL-producing among MDR *E. coli* isolates from various clinical samples at "Hôpital national de Niamey" and "Hôpital national lamordé", Niger.

MATERIALS AND METHODS

Study design and site

The present prospective study was conducted on routine specimens received at the bacteriology laboratory of"Hôpital National de Niamey" and the "Hôpital National Lamordé", with a capacity of 800 and 500 beds, respectively. Bacterial isolates that were resistant to third generation of cephalosporin were collected from March 2014 to June 2014 and then from October 2014 to June 2015 (13 months) simultaneously in two hospitals. They were isolated during diagnosis analysis of biological specimens. Different clinical specimens such as blood, pus, urine, stool, and vaginal swab were collected.

Isolation and identification of *E. coli*

Bacterial isolates were obtained from various clinical specimens. Stool samples were inoculated on eosin methylene blue agar (EMB, Merseyside UK), urine samples on cystine lactose electrolyte deficient agar (CLED, Liofilchem), pus and vaginal swabs were cultured on blood and chocolate agar and were incubated at 37°C for 18 to 24 h. Blood samples were inoculated and incubated in culture bottles with Bact/Alert 3D 60 Biomérieux at 37°C. The isolates were identified according to the procedures described by Cheesbrough et al. (2005) and were confirmed using gallery API 20 E system (Biomérieux, France). The clinical isolates were preserved at −70°C for further analysis.

Antibiotic susceptibility testing

Antibiotic susceptibility was determined using the disk diffusion method on Mueller-Hinton (MH) agar plates (Liofilchem, Italy) according to the recommendations of "Comité de l'Antibiogramme de la Société Francaise de Microbiologie" (CA-SFM, 2012). The following antimicrobials were tested: amoxicillin (25 µg), amoxicillin + clavulanic acid (20 +10 µg), cephalothin (30 µg), cefoxitin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxon (30 µg), ofloxacin (5 µg), nalidixic acid (30 µg), aztreonam (30 µg), amikacin (30 µg), gentamicin (15 µg), ciprofloxacin (5 µg), nitrofuran (300 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg) and imipenem (10 µg). Quality control was done using *E. coli* ATCC 25922. Multidrug resistant *E. coli* was defined as resistance to at least three classes of antibiotics.

Detection of extended-spectrum beta-lactamase (ESBL) by DDST

All MDR *E. coli* isolated were screened for ESBL. The double-disk synergy test (DDST) was performed for the phenotypic detection of ESBL producers according to the CA-SFM recommendations, using ceftazidime (30 µg), aztreonam (30 µg), cefotaxime (30 µg) disks and were placed 25 mm (center to center) from the amoxicillin/clavulanic acid (20/10 µg) disk on Mueller–Hinton agar (CA-SFM, 2012). After inoculation, the plates were incubated at 37°C; the presence of a keyhole effect was recorded 24 h after incubation. The DDST was performed in parallel to the antibiogram.

Detection of extended-spectrum beta-lactamase by DDST using cloxacillin

Cloxacillin test was performed for MDR isolates naturally producing inducible céphalosporinase (AmpC). The production of ESBL was inferred by a synergy image as previously described (Drieux et al., 2008). From July 2014 to September 2014 and then from July 2015

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Table 1. Global antimirobial susceptibility of MDR *E. coli*.

Antibiotics	Resistance percent (%)	Susceptible percent (%)
Amoxicillin	100	0
Amoxicillin/ Clavulanic	93.1	6.9
Cephalothin	98.2	1.8
Cefoxitin	35.9	64.1
Cefotaxime	92.6	7.4
Ceftazidime	97.2	2.8
Ceftriaxone	83.9	16.1
Ofloxacin	77.4	22.6
Nalidixic Acid	91.2	8.8
Amikacin	10.6	89.4
Gentamicin	36.9	63.1
Ciprofloxacin	82.9	17.1
Aztreonam	77.4	22.6
Nitrofuran	21.7	78.3
Trimethoprim/sulfamethoxazole	95.4	4.6
Imipenem	1.4	98.6

to September 2015, all MDR *E. coli* that were ESBL negative for DDST, were tested by DDST using cloxacillin.

The DDST was also performed with cloxacillin (200 µg/ml) containing MH agar plates (bioMérieux). In the situation of the absence of growth of *E. coli* on MHA with cloxacillin at a concentration of 200 µg/ml, cloxacillin at a concentration of 100 µg/ml was used (Drieux et al., 2008).

Ethical consideration

All isolates obtained were from biological specimens on clinical routine examinations of patients, with the authorization of the laboratory director and hospital director.

Data analysis

Data were analyzed using Excel, Microsoft® Office 2013. Chi square was used to determine the statistical significance of the data. Statistical significant difference was considered with a p-value < 0.05.

RESULTS

Antimicrobial susceptibility testing

During the study period, two hundred and seventeen (217) MDR *E. coli* were isolated from the two hospitals. High resistance to beta-lactams was observed, mainly with ampicillin (100%), amoxicillin + clavulanic acid (93.1%), cephalothin (98.2%), cefotaxime (92.6%), ceftazidime (97.2%) and ceftriaxone (83.9%) as compared to quinolone with ofloxacin (77.4%), ciprofloxacin (84.9%) and nalidixic acid (91.2%). Resistance to the nobactams was 77.4% to aztreonam, and the sulfonamides was 95.4% to trimethoprim-sulfamethoxazole (Table 1).

Table 2. Prevalence of ESBL among MDR *E. coli.*

	Positive ESBL		Negative ESBL
	DDST	DDST + cloxacillin	
Isolates number	49	8	160
Prevalence (%)	26,3		73,7

Prevalence of ESBL among MDR E. coli

Among the 217 MDR *E. coli* isolated, screening for ESBL production showed a global prevalence of 26.3% (57) as shown in Table 2. The difference between percentages of ESBL-producing (26.3%) and non-ESBL-producing (73.7%) *E. coli* was highly significant (P = 0.0001). The DDST showed 22.6% (49) of ESBL *E. coli* prevalence. Those *MDR E. coli* (168) that were negative to ESBL were tested with DDST using cloxacillin. Of these isolates, ESBL prevalence of 4.8% (9) was observed (P = 0.0013). This indicated a significant difference between the two methods.

Prevalence of ESBL E. coli according to demographic characteristic of the studied patients

The demographic characteristic of the studied patients is summarized in Table 3. Of the 217 MDR *E. coli,* 150 (69.1%) were isolated from outpatients, and 67 (30.9%) from inpatients (P= 0.0002). However, ESBL prevalence of 25.3 and 28.4% from outpatients and inpatients were

Table 3. Prevalence of ESBL *E. coli* according to demographic characteristic of the studied patients.

Demographic variables	MDR *E. coli* n (%)	ESBL *E. coli* n (%)
Gender		
Male	118 (54.4)	29 (24.6)
Female	99 (45.6)	28 (28.3)
Age group (years)		
≤ 5	113 (52.1)	28 (24.8)
6 - 25	13 (6.0)	3 (23.1)
26 - 45	21 (9.7)	8 (38.1)
46 - 65	26 (12.0)	3 (11.5)
> 65	18 (8.3)	9 (50.0)
ND	26 (12.8)	6 (23.1)
Patients		
Outpatients	150 (69.1)	38 (25.3)
Inpatients	67 (30.9)	19 (28.4)
Hospital		
HNN	41 (89)	9 (10.1)
HNL	59 (128)	48 (37.5)

ND: Not determined.

observed respectively (P= 0.78). The distribution of ESBL producers based on gender indicates that women had a higher prevalence rate of 28.3% than men, 24.6% (P= 0.78). There was no significant difference between the gender distributions and the source patients. Otherwise, samples were collected from patients ranging in age from 1 month to over 65 years. The highest prevalence (50%) of ESBL was observed among the age group over 65 years followed by the age group of 26 to 45 years (38.1%), then the age group under five years (24.5%), age group 6 to 25 (23.1%) and the least in age group of 46 to 65 years (11.5%). Low prevalence of ESBL *E. coli* was observed in “Hôpital National de Niamey” with 10.1%; as compared “Hôpital National Lamordé” (37.5%) (p= 0.0001). This difference was significant among the two hospitals.

Prevalence of ESBL E. coli according to biological specimens

Most of the MDR *E. coli* were isolated from the urine samples (67.3%) followed by stool samples (26.3%) as shown in Table 4. However, only 26.7 and 26.3% of urine and stool isolates, respectively were ESBL-producers. On the other hand, in spite of their small number, 25% of isolates from pus and blood samples were ESBL producers.

Table 4. Prevalence of ESBL *E. coli* according to biological specimens.

Biological samples	MDR *E. coli* % (n)	ESBL *E. coli* % (n)
Urine	67.3 (146)	26.7 (39)
Stools	26.3 (57)	26.3 (15)
Pus	3.7 (8)	25 (2)
Blood	1.8 (4)	25 (1)
Vaginal swabs	0.9 (2)	0 (0)

DISCUSSION

The number of infections due to ESBL *E. coli* is increasing, especially in African countries (Manyahi et al., 2014). In this study, the authors investigated the prevalence of ESBL production by MDR *E. coli* isolated from clinical samples sent to the two main hospitals of Niamey. The antimicrobial susceptibility tests showed an important level of resistance of antibiotics classes. Thus, for beta-lactams classes, resistance frequency of 93.1% was observed for amoxicillin-clavulanate, 92.6% for cefotaxim and 97.2% for ceftazidim. Similar results have been reported in Nigeria with a prevalence of 89.71% for amoxicillin-clavulanate 79.47% for cefotaxim and ceftazidim 41.03% (Odumosu and Akintimehin, 2015). On the other hand, co-resistance was shown for different antibiotics such as ofloxacin (77.4%), ciprofloxacin (84.9%) and nalidixic acid (91.2%). Similar results were observed in Ivory Coast with a prevalence of 70.2% for ciprofloxacin and 76.8% for nalixidic acid (Guessennd et al., 2008). Such level of resistance could be due to abusive prescription of antibiotics by professionals of health care without prior laboratory investigations or parallel care at home, self-medication and also the use of street drugs which is very spread in Africa (Yandai et al., 2014). Nevertheless, this study showed a susceptibility of 95.4% for imipenem, this frequency was similar to published data in Burkina Faso, which showed a susceptibility of 100% for imipenem (Sanou et al., 2015).

In all the 217 MDR *E. coli* isolated in this study, ESBL prevalence of 26.3% was observed. Similar prevalence was found in some African countries such as in Benin (22%) (Ahoyo et al., 2007), Nigeria (20%) (Onwuezobe and Orok, 2015), Niger (30.9%) (Woerther et al., 2011) and Chad (20.09%) (Yandaï et al., 2014). As compared to previous studies, the current study results are lower than that observed in Burkina Faso (38.3%) (Dembélé et al., 2015) and Senegal (52%) (Lo et al., 2014). However, these results are higher than that observed in Tanzania (15.1%) (Mshana et al., 2016) and the Libyan community (13.4%) (Ahmed et al., 2014). This wide variation in prevalence was probably due to differences in type of samples collected.

The study showed the prevalence of ESBL producers

based on gender and indicated that females had a higher prevalence rate (28.3%) than males (24.6%). No differences were apparent between ESBL-producing *E. coli* with gender distribution (Akanbi et al., 2013). However, contradictory observations were found in Nigeria (Yusuf et al., 2013).

In this study, the majority of ESBL-producing *E. coli* was isolated from inpatients (28.4%) as compared to outpatients (25.3%). The rates of ESBL-producing *E. coli* were higher among inpatients (22.82%) than the outpatients (18.11%) as reported in Chad (Yandai et al., 2014).

Among the patients studied, the highest prevalence was observed in age group over 65 years (50%) followed by age group 45 to 65 years (38.1%) and the age group under 5 years (24.8%). Higher median age was observed among individuals colonized with ESBLs as reported from Tanzania (Mshana et al., 2016). Although, previous study in Guinea-Bissau showed that ESBL *E. coli* prevalence was high in all age groups, among the youngest 27% were carriers in the ages 0 to 3 months. This indicates that colonization with ESBL-producing bacteria often occurs early in life in this population (Isendahl et al., 2012).

This study showed that MDR *E. coli* were isolated from a variety of clinical samples. Thus, it was found that urine samples had the highest proportion of ESBL (26.7%) followed by stool samples (26.3%). However, the same prevalence (25%) was observed from blood and pus samples. The major ESBL *E. coli* producer (18.2%) was isolated from urine samples (Raut et al., 2015). Ouedraogo et al. (2016) found that blood cultures had the highest proportion of ESBL isolates. In previous study, the distribution of ESBL producers was most prevalent in blood (22.2%) and urine samples (17.6%). This was followed in that order by stool (15.8%), urogenital swabs (14.3%) and wound swab (13.5%) while the least prevalence was observed in ear swab specimens (3.2%) (Yusuf et al., 2013). This difference of ESBL producers among clinical source could be due to lower number of some samples.

Therefore, infection control strategies, with the rational use of antibiotics could be an important factors to reduce the spread of ESBL. Further investigations, including molecular characterization of different ESBL was necessary to understand the spread of resistant bacteria.

Conclusion

This study demonstrated the high occurrence of ESBL produced by *E. coli* isolated in two hospitals of Niamey. All the age groups were concerned with high resistances of the ESBL-producing isolates to antibiotics classes. Urine and stool samples had a higher prevalence of ESBL producers in Niamey. Hence, it was necessary to develop a nosocomial infection control and antimicrobial surveillance system in all health centers in order to avoid emergence and clonal spread of ESBL *E. coli*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Serological detection of bacterial pathogens associated with rotted potato tubers

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Studies were conducted in the Department of Plant Pathology, College of Agriculture, Orissa University of Agriculture and Technology, Odisha, India in 2010-2011 on serological detection methods for identification of bacterial pathogens associated with rotted potato tubers. One hundred and two rotten potato tubers of KufriJyoti variety were collected from freshly harvested lot of All India Co-ordinated Potato Improvement Project, Orissa University of Agriculture and Technology. Tubers were cut into two equal halves and categorized into 6 groups, on the basis of internal symptoms exhibited, that is, (A) cut tubers showing brownish discolouration along the vascular region, (B) cut tubers showing brownish discolouration along the vascular region with soft rotten cavities filled with whitish ooze, (C) cut tubers showing brownish blackish discolouration along the vascular region and soft rotten tissues extending towards centre, (D) cut tubers showing soft rotten tissues extending towards centre without brownish discolouration, (E) cut tubers showing soft rotten tissues extending towards centre with brownish black discolouration, (F) cut tubers showing dry tissues with cavities surrounded by soft tissues. The association of two bacterial species was assayed following tube agglutination test using the known antiserum for each bacterium, *Ralstonia solanacearum* and *Pectobacterium carotovorum*. It was revealed that *R. solanacearum* could be associated exclusively with 54.10% of diseased tubers with symptom Category–A. No other bacteria could be detected from the rest of the samples belonging to the said category. Similarly, exclusive association of *P. carotovorum* could be detected in 87.50% of the rotten tubers with symptom Category–D. In the symptom categories B, C, E and F, both test bacteria were found to be associated either singly or as mixture. Least bacterial infection due to *P. carotovorum* (12.5%) was observed in symptom Category–F. It is a very quick detection method which can reveal the percentage of bacterial pathogen association after two hours of testing.

Key words: Rotten potato tubers, bacteria, bacterial pathogen, serological detection, *Ralstonia solanacearum, Pectobacterium carotovorum.*

INTRODUCTION

Rotting of potato tubers is commonly noticed at the time of harvest, in storage at country stores and also at cold stores. The rotten tubers exhibit brown rot, soft rot and mixed symptoms. Accurate identification of the causal pathogen is necessary because management strategy is almost different for different organisms. The usual method of identification of the causal bacterial plant pathogen involves isolation, pathogenicity test followed by Gram staining, microscopic studies, growing them on selective medium, a series of biochemical tests and carbohydrate

Table 1. Different types of tuber rotting symptoms examined for detection of association of test bacterial species following tube agglutination test.

Category	Symptoms observed	No. of tubers studied
A	Cut tubers showing brownish discoloration along the vascular region	24
B	Cut tubers showing brownish discoloration along the vascular region with soft rotten cavities filled with whitish ooze	16
C	Cut tubers showing brownish blackish discoloration along the vascular region and soft rotting of tissues extending towards the centre	24
D	Cut tubers showing soft rotten tissues extending towards the center without brownish discolouration.	8
E	Cut tubers showing soft rotten of tissues extending towards the centre with brownish black discoloration surrounded by corky tissues.	22
F	Cut tubers showing dry tissues with cavities surrounded by corky with soft tissues round it	8

tests and studies at molecular levels followed in different countries like Japan and Mauritius (Rodney et al., 2010; Harita et al., 2010; Suga et al., 2013). Molecular level of isolation and characterization of the phytopathogenic bacteria has recently gained wide acceptance (Tamura et al., 2011; Zhou et al., 2012). Rahman et al. (2012) characterized soft rot bacterial strain of potato following physiological and biochemical tests such as (i) potato soft rot test; (ii) Gram reaction test; (iii) glucose fermentation, oxidase reaction; (iv) catalase test and (v) gelatine liquefaction test, nitrite test, indole test and lecithinase test. Ravari et al. (2011) isolated forty strains from macerated potato tubers and water soaked lesions of some ornamental plants in north parts of Iran, proved pathogenicity in their respective hosts. The causal organisms were identified as *Pectobacterium* spp. based on their physiological and biochemical assays and confirmed by species and subspecies specific PCR and RFLP analysis of 16S-23S intergenic transcribed spacer region. Dhital et al. (2001) characterized the strains of *Ralstonia solanacearum* (the causal agent of bacterial wilt disease from Nepal and Thailand) on the basis of pathogenicity, biochemical/physiological and serological tests. Among different recent techniques, one is detection of quorum sensing molecules in *R. solanacearum* which may be responsible for virulence (Kumar et al., 2016). Similarly, molecular methods of characterization in Potato virus Y was studied in different countries by different workers (Chikh-Ali et al., 2016). Isolation and characterization of *R. solanacearum* was studied by several workers (Lemessa and Zeller, 2007; Alvarez et al., 2010; Tamura et al., 2011).

Both bacteria (*R. solanacearum* and *P. carotovorum*) caused rotting at harvest. *P. carotovorum* continues to enhance rotting in transit and storage and spread easily by contact. Quick detection will help in adopting adequate management practices at earliest possible time and the loss can be reduced subsequently. Among different methods, the serological detection method is also the most accurate, simple and quick technique. By using specific antiserum, the occurrence of particular race or strain of a particular plant pathogenic bacteria and virus can be studied easily and also at earliest time and further studies can be conducted. In the present study, serological detection methods were followed for identification of bacteria associated with rotted potato tubers.

MATERIALS AND METHODS

One hundred and two apparently rotten potato tubers were collected from the harvested lot of All India Co-ordinated Potato Research Project, Orissa University of Agriculture and Technology, Bhubaneswar and cut into two equal halves. On the basis of internal rotting symptom, the tubers were categorized into six different rotting groups. The association of two bacterial species were assayed following tube agglutination test using the known antiserum for each bacteria: *R. solanacearum* and *P. carotovorum*.

The rotting symptoms observed were categorized into 6 groups namely; (A) cut tubers showing brownish discoloration along the vascular region (24), (B) cut tubers showing brownish discolorations along the vascular region with soft rotten cavities filled with whitish ooze(16), (C) cut tubers showing brownish blackish discolorations along the vascular region and soft rotten tissues extending towards centre (24), (D) cut tubers showing soft rotten tissues extending towards the center without brownish discoloration (8), (E) cut tubers showing soft rotten tissues extending towards the centre with brownish black discoloration (22), (F) cut tubers showing dry tissues with cavities surrounded by soft tissue (8) as presented in Table 1. Each tuber collected from experimental field was carefully washed in tap water using a soft brush. Care was taken not to damage the skin of tuber or disturb the disease symptom. Then it was blot dried and examined for characteristic symptom developed. Based on the symptom, the tubers were separated. Individual tubers in each symptom group were tested for association of test bacteria following

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Table 2. Serological detection of *R. solanacearum* and *P. carotovorum* associated with different categories of tuber rotting.

Category	Symptoms observed	Percent of tubers under different category (%)	*Ralstoniasolanaceaurum* (%)	*Pectobacteriumcarotovorum* (%)	Mixed infection with the test bacteria (%)	Non-detection of test bacteria (%)
A (Figure 1)	Cut tubers showing brownish discolouration along the vascular region	23.52	54.10	0.00	0.00	45.90
B (Figure 2)	Cut tubers showing brownish discolouration along the vascular region with soft rotten cavities filled with whitish ooze	15.68	56.20	31.20	12.60	0.00
C (Figure 3)	Cut tubers showing brownish blackish discolouration along the vascular region and soft rotting of tissues extending towards the centre	23.52	0	0.00	83.30	16.70
D (Figure 4)	Cut tubers showing soft rotten tissues extending towards the centre without brownish discolouration.	7.84	0	87.50	0.00	12.50
E (Figure 5)	Cut tubers showing soft rotten of tissues extending towards the centre with brownish black discolouration surrounded by corky tissues.	21.56	22.70	45.40	31.90	0.00
F (Figure 6)	Cut tubers showing dry tissues with cavities surrounded by corky with soft tissues round it	7.84	0	12.50	0.00	87.50

modified tube agglutination test. Completely rotten portion of individual tuber in a group was separated and the apparently healthy tissues adjacent to disease portion were cut into small pieces of about 1.0 cm size. Five cut pieces were transferred into 0.5 ml of sterile water taken in a culture tube. It was kept under laboratory condition for 30 min for bacterial oozing.

For detection of test bacterial species present in the bacterial suspension of each diseased tuber, sample was carried out following tube agglutination test. For each sample, two agglutination tubes were taken for the two known antisera. 0.5 ml of 10.0% diluted solution of each known antiserum was transferred into one tube and the other known anti serum to the second tube and leveled properly. To each of the two tubes, 0.5 ml of bacterial suspension collected from one diseased sample was transferred and mixed thoroughly using Pasteur pipettes. The process was repeated for subsequent disease samples. The tubes were incubated in a hot water bath maintained at 37±1°C for 2 h. Formation and deposition of precipitate at the base of the tube indicated positive reaction on the basis of positive reaction against the known antiserum.

RESULTS

Results revealed that out of total diseased tubers, 23.52% of the tubers exhibited symptom categorized with brownish discoloration along the vascular region without any soft rotting of tissues (Category–A) (Table 2 and Figure 1). About 15.68% of tubers exhibited symptom with brownish discoloration along the vascular region with soft rotten cavities filled with whitish ooze (Category–B) (Figure 2). It was closely followed by the Category–C (23.52%) (Figure 3) with brownish black discoloration along the vascular region with soft rotting of tissues extending

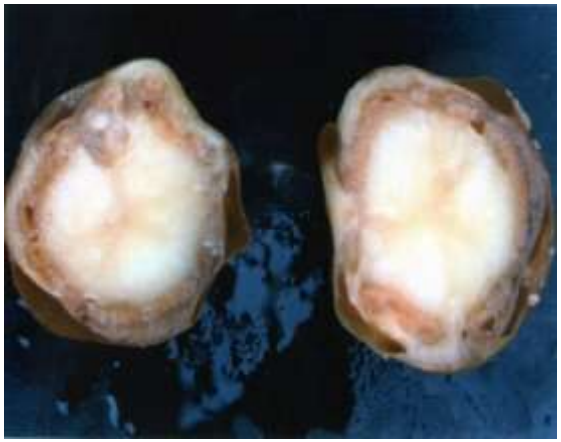

Figure 1. Symptom-A (cut tubers showing brownish discoloration along the vascular region).

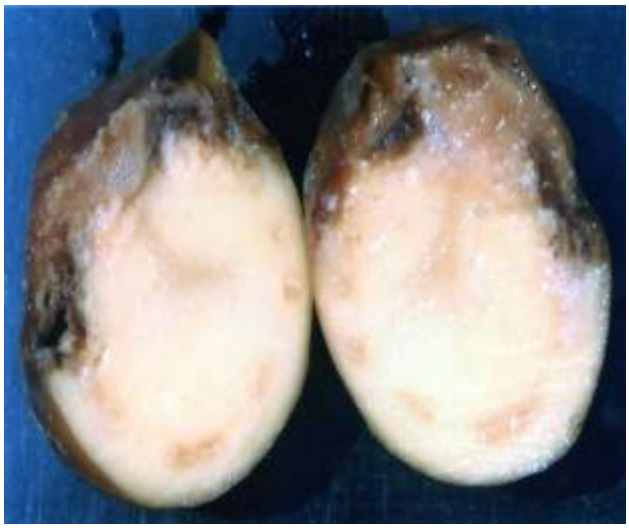

Figure 2. Symptom-B (cut tubers showing brownish discolouration along the vascular region with soft rotten cavities filled with whitish ooze).

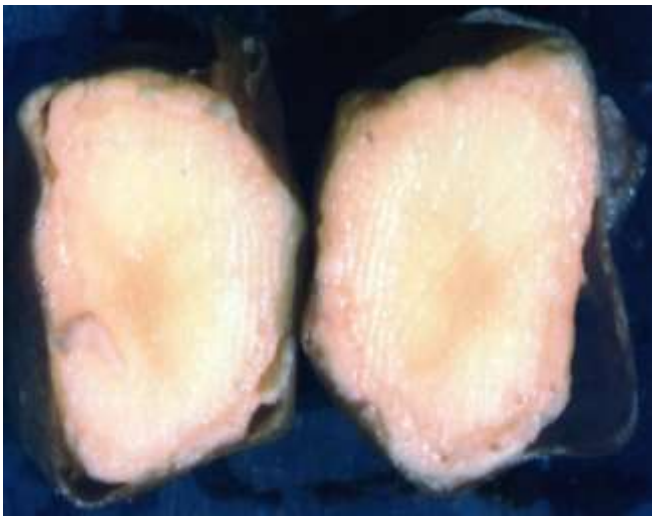

Figure 4. Symptom-D (cut tubers showing soft rotten tissues extending towards the centre without brownish discolouration).

Figure 3. Symptom-C (cut tubers showing brownish blackish discoloration along the vascular region and soft rotting of tissues extending towards the centre).

Figure 5. Symptom-E (cut tubers showing soft rotting of tissues extending towards the centre with brownish black discoloration surrounded by corky tissues).

towards the centre. About 7.84% tubers exhibited symptom both in categories– D and F each (Figure 4). In Category–D (Figure 4), the tubers showed soft rotten tissues extending towards the center without brownish discoloration and in Category–F (Figure 6), the tuber exhibited dry cavities surrounded by corky layer with soft tissues round it. In Category–E (Figure 5), 21.56% of the tubers showed soft rotten tissues extending towards the centre with brownish black discoloration.

R. solanacearum could be found associated exclusively in 54.10% of the diseased tubers of symptom Category–A. No other bacteria could be detected from the rest of the samples belonging to the said category. Similarly, exclusive association of *P. carotovorum* could be detected in 87.50% of the rotten tubers of symptom category–D. In the symptom category B and E, both test bacteria were found to be associated either singly or as mixture. However, maximum infection of 56.20% was caused by *R. solanacearum* in symptom Category–B followed by *P. carotovorum.*

Similarly *P. carotovorum* was found to be associated with maximum 45.40% of tuber infection as given in Category–E. Mixed infection of both bacteria could be detected in 31.9% of diseased tubers as per Category-E. Only 12.50% of tubers found were associated with *P. carotovorum* in symptom Category-F and no bacterial infection was found in the rest 87.50% of

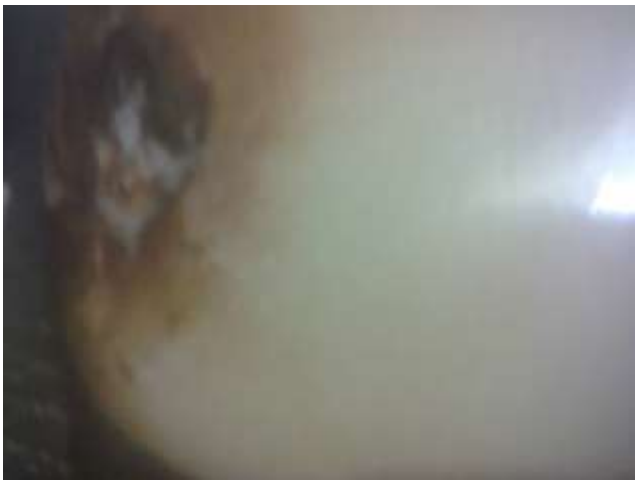

Figure 6. Symptom-F (cut tuber showing dry tissues with cavities surrounded by corky with soft tissues round it).

tubers.

DISCUSSION

Brown rot (*R. solanacearum*) and soft rot (*P. carotovorum*) are the two major devastating diseases of potato at harvest in Odisha India. Present studies followed sero-diagnosis methods and revealed that most tubers rotted due to *P. carotovorum* followed by *R. solanacearum.* Mixed infection by both bacteria was also recorded.

Serological detection methods were also used by different workers around the globe in different crops in addition to strains on potato. Khder et al. (2014) studied in detail, the serological characteristics of *Erwinia carotovora* isolated from fields in Egypt. Silveria et al. (2002) worked on production of antisera of soft rotting *Pectobacteria*. Improved monospecific serum technology was also used for diagnosis of *E. Amylovora* (Bokszan and Sadlak, 2001). Serological methods was successfully quantified for the potato seed contamination by *E. carotovora sub* sp. a*troseptica* (Perombelon and Hyman, 1995). Serological studies were done with 11 isolates of *Erwinia carotovora* sub sp. *atroseptica* (Mierzwa et al., 1979). Tanii and Akaii (1975) demonstrated black leg was caused by serologically specific strain of *E. carotovora*. Dobias (1973) detected the serological relationship of strains of *E. carotovora*. Prez (1962) worked serologically on identification of *Pseudomonas solanacearum*. Several workers also adopted serological methods in studies of viral and fungal diseases. Galvino-Costa et al. (2012) worked together on molecular and serological typing of Potato virus Y isolates from Brazil. Karasev et al. (2011) studied serologically, the genetic diversity of the ordinary strain of Potato virus Y (PVY) and origin of recombinant PVY strains. Serological properties of ordinary and necrotic isolates of PVY were taken into account to survey the occurrence for both isolates in the U.S seed crop (Karasev et al., 2010). Baldauf et al. (2006) worked on biological and serological properties of Potato Virus Y isolates in Northeastern United States potato in New York. Mahmoud et al. (2010) followed serological method for detection of *Phytophthora infestans* in infected symptomatic and asymptomatic potato tissues (leaves and tubers) and could provide important interaction and disease development. Llave et al. (1999) followed serological analysis for coat protein sequence determination of potato virus Y pepper pathotypes. Sukla et al. (1988) compared four strains of potato viruses Y, that is, PVY-D, PVY-10, PVY-18 and PVYPVY-43 on the basis of their biological, serological and coat protein structural properties. McDonald and Singh (1996) established the serology of the isolates of PVY that share properties with both PVY^N and PVY^0 strain groups. De Boer et al. (1979) categorized sero groups of *E. carotovora* potato strains. Dobias (1973) detected the serological relationship of strains of *E. carotovora*. Serological detection methods has already been followed in other crops, that is, poplar (Zhang et al., 2001), wheat (Shneider et al., 1978) and also in hyacinth and calla (Kabashna, 1977). Hence, by using specific antiserum, the occurrence of particular race or strain of a particular plant pathogenic bacteria and virus in potato can be studied easily. Quick detection technique is very much essential for effective management of the disease in stored condition and also in field during growing and harvesting stage.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Atypical manifestation in infection by methicillin-resistant *Staphylococcus aureus* carrier SCC*mec* IV and Panton-Valentine Leukocidin-producer in experimental sepsis model

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***Staphylococcus aureus* is considered an infectious agent of great clinical importance, responsible for many different types of infection. Strains of methicillin-resistant *Staphylococcus aureus* (MRSA), Panton-Valentine leukocidin producers, are considered more invasive, presenting clinical sequelae related to abscesses and infection in skin and soft tissues. The use of invasive techniques in hospital environment, such as the introduction of intravascular catheter in immunocompromised patients, has contributed to this microorganism spreading through the bloodstream, causing bacteremia, necrotizing pneumonia and increasing the number of septic patients in intensive care units with high mortality. In this report, atypical infections in Swiss mice using experimental model of sepsis was presented.**

Key words: methicillin-resistant *Staphylococcus aureus* (MRSA), mice infection, Panton-Valentine Leukocidin.

INTRODUCTION

Staphylococcus aureus is part of the natural microbiota of skin and nasal cavities (Kim et al., 2014; Lowy, 1998). Its capacity of colonization and pathogenicity is due to its virulence factors, important in adhesion and evasion of the host's immune system (Otto, 2010; Tavares, 2002). This microorganism is the most common agent causing pyogenic infections, having as primary site, the skin, and through bacteremia can infect several other organs (Andriolo, 2005; Boles and Horswill, 2008). The pathological features of *S. aureus* infections are due to

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the production of toxins, determinants factors of its virulence (Bukowski et al., 2010; Kim et al., 2014; Novick et al., 2010) which are commonly associated with purulent lesions and abscesses due to infiltration of neutrophils at infected site (Cheng et al., 2009; Kim et al., 2014).

The erroneous and constant use of antimicrobials in animals and humans led to selection of strains resistant to β-lactams and Cephalosporins being called methicillin-resistant *Staphylococcus aureus* (MRSA) (DeLeo and Chambers, 2009; Kim et al., 2014). By the acquisition of the chromosomal gene of *mec*A resistance which encodes structural modifications in receptor surface protein for β-lactams, there is promotion of dissemination of this microorganism in a hospital environment and making it difficult to treat infections (Atshan et al., 2012; Berger-Bachi and Rohrer, 2002).

An important cytotoxin secreted by *S. aureus* disseminated worldwide (Diep and Otto, 2008) is the Panton-Valentine leukocidin (PVL), commonly associated with community strains containing type IV staphylococcal cassette chromosome *mec* (SCC*mec*) (Diep et al., 2006; Vandenesch et al., 2003). Increasingly, community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains producing of PVL have been isolated from skin abscesses, soft tissue infection and necrotizing pneumonia in hospital settings (Gillet et al., 2002; Lina et al., 1999), being a major public health problem and more harmful and immunological weakness of infected patients. This leukocidin targets the polymorphonuclear leucocytes and macrophages. In high concentrations, this molecule forms pores in membrane, altering the permeability and allowing cations to enter (Ca^{2+}) causing degranulation and subsequent cytolysis, and in low concentration, promotes apoptosis by binding to the mitochondrial membrane, resulting in the release of oxygen in the reactive form (Genestier et al., 2005; Boyle-Vavra and Daum, 2007). The appearance of these strains in hospital infections may be related to preexisting colonization of the patient, which finds a port of entry during invasive surgical procedures (Enright et al., 2002; Maree et al., 2007), also through direct manual contact of health professionals with open lesions in infected patients in postoperative period. Because it is an easily transmitted microorganism, the eradication of staphylococcal infections in hospitals is becoming increasingly difficult and may be endemic in some countries (Michelim et al., 2005). For the treatment of infections caused by methicillin-resistant *S. aureus* from producer *pvl* (MRSA *pvl* (+)), the antimicrobials that proved to be effective were Vancomycin, Daptomycin, Linezolid and Teicoplanin (Lima et al., 2011; Kim et al., 2014; Liu et al., 2011).

This study aims to evaluate the pathogenicity of MRSA strains isolated from nasal colonization and infection in humans, and may help in research using experimental models that replicate the pathophysiology of the disease in humans.

MATERIALS AND METHODS

Experimental animals

Twenty-five (25) Swiss inbred mice were used in this study. The animals were young adults, six weeks of age, male weighing approximately 34 g and specific pathogenic-free (SPF). The animals mentioned were part of an experimental study approved by the Ethics Committee on Animal Research of the Pro-Rectory of Research and Postgraduate from Federal Fluminense University under the registration number, 439/2013.

Each experimental group was divided according to genotypic characteristics and colonization sites from which bacterial strains were isolated in humans. The animals were kept in collective and ventilated cages containing five animals in each group, which received commercial diet and filtered water *ad libitum*. Animals were exposed to light-dark cycles, at the temperature of 21 to 22°C (± 2) and 50 to 55% humidity.

Bacterial samples

Microbiological samples are part of collection of the Laboratory of Molecular Epidemiology and Biotechnology, Rodolpho Albino University Laboratory, Federal Fluminense University. Samples were preserved in brain heart infusion (BHI) medium containing 10% of glycerol and frozen at -80°C.

All bacterial samples were phenotypically tested in order to identify *S. aureus* using Gram staining, colonial morphology, fermentation of mannitol-salt agar (Zimbro et al., 2009), catalase production (Murray et al., 2007) and coagulase production (McDonald and Chapin, 1995). Thereafter, the species was confirmed by performing polymerase chain reaction (PCR) for 442 bp chromosomal DNA fragment, as protocoled by Martineau et al. (1998).

Methicillin resistance was identified using PCR for *mec*A gene according to the protocol of Oliveira and Lencastre (2002). The production of PVL as virulence factor was confirmed by *luk*F-PV and *luk*S-PV genes as established by Lina et al. (1999).

Bacterial samples selected for this study exhibited the following characteristics: methicillin-susceptible and PVL non-producing strains isolated from nasal colonization, *pvl* (-) MSSA; methicillin-susceptible and PVL-producing strains isolated from nasal colonization, *pvl* (+) MSSA; methicillin-resistant and PVL-producing strains isolated from peripheral blood of patients with severe pulmonary infection, *pvl* (+) MRSA.

Induction of infection

Bacterial colonies were cultivated for 24 h on trypticase soy agar (TSA) and suspended in sterile test tube containing 1000 µL of saline solution (NaCl 0.9%). Subsequently, serial dilutions were performed in order to obtain a density 1.0×10^7 colony forming units (CFU/mL).

The animals were anaesthetized through inhalation of Isoflurane FORANE® (2-chlorine-2-(difluorometoxy)-1.1.1-trifluor-ethane) in close campanula (Kiedrowski and Horswill, 2011), 50 µL of the bacterial suspension were intravenously inoculated through the tail vein, except in the control group (CG), which received the same volume of sterile saline solution. Animals were maintained in their respective cages for 96 h.

Histopathology

After 96 h, the animals were euthanized by overdose of Isoflurane

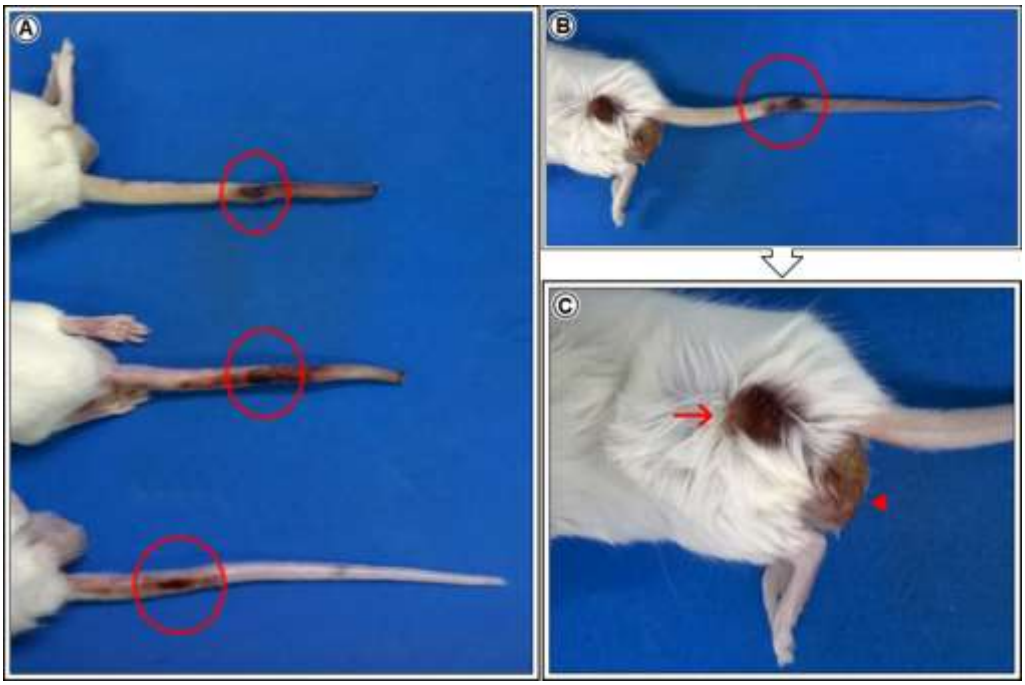

Figure 1. Infection in tail, skin and testicles. (A) Tails of mice inoculated with MSSA *pvl* (-) presenting ulceration and hyperemia, distal region with necrosis and region near base edematous. (B) Tails of mice inoculated with MSSA *pvl* (+) with ulceration and hyperemia, distal region with necrosis and region near the base edematous. (C) Posterior limb with skin infection (arrow), testicles with intense edema and hyperemia (arrowhead).

FORANE® (2-chlorine-2-(difluorometoxy)-1.1.1-trifluor-ethane). The eyeball was extracted by applying a pressure using tweezers around the orbital cavity. The samples were placed in cassettes and stored in 10% formaldehyde with pH between 0.6 and 0.7 during 48 h for the preparation of histological slides. Posteriorly, submitted to the processes of dehydration, diafanization and inclusion in paraffin, for the confection, 3-µm-thick sections were cut on a microtome (LAB-MR500), fixed on slides and stained with hematoxylin and eosin (H&E). The slides were observed in optical microscope (model LX 500) and photographed using iVm 5000 camera and ProgRes capture Pro 2.7 program to the description of inflammatory processes.

RESULTS

Among the results obtained, it was possible to observe pathological alterations not yet reported in an experimental model of systemic infection by this microorganism in mice. Three animals inoculated with β-lactam susceptible strain and not producing PVL: MSSA *pvl* (-), one animal inoculated with a strain producing PVL: MSSA *pvl* (+), and one animal inoculated with bacterial strain resistant to β-lactams and producer of PVL: MRSA *pvl* (+) isolated from peripheral blood in a patient afflicted by severe pulmonary infection; after 72 h of infection died of septic, with formation of ulceration and hyperemia at the site of inoculation (tail). The infectious process spread throughout the tail, causing end loss in distal region due to necrosis. In the region close to base of tail, pallor characteristic of edema was found (Figure 1A, B and Figure 2A). Only the animal inoculated with MSSA *pvl* (+) strains, presented a circular infectious region with hair loss and hyperemia on skin of right hind limb. Strong edema with hyperemia was also observed in both testes (Figure 1C). An unusual occurrence observed in the animal inoculated with MRSA *pvl* (+), was intense palpebral edema, with infection composed of purulent material, thick and yellowish covering the sclera in both eyes (Figure 2B). The histopathological analysis of the ocular globe revealed the presence of fibrinous inflammatory exudate adhered to the sclera, with cellular debris of necrotic tissue in the epithelium and some regions with leukocyte invasion (Figure 2C). CG animals did not present any anatomical alteration or inflammatory reaction at the inoculation site.

DISCUSSION

The observation of dermonecrosis in the skin of the right hind limb, tail necrosis and eye infection, using a model of systemic infection through the intravenous route, have not yet been reported in studies using a similar infection model. However, there are reports of same pathological processes in infections models skin using rabbit having

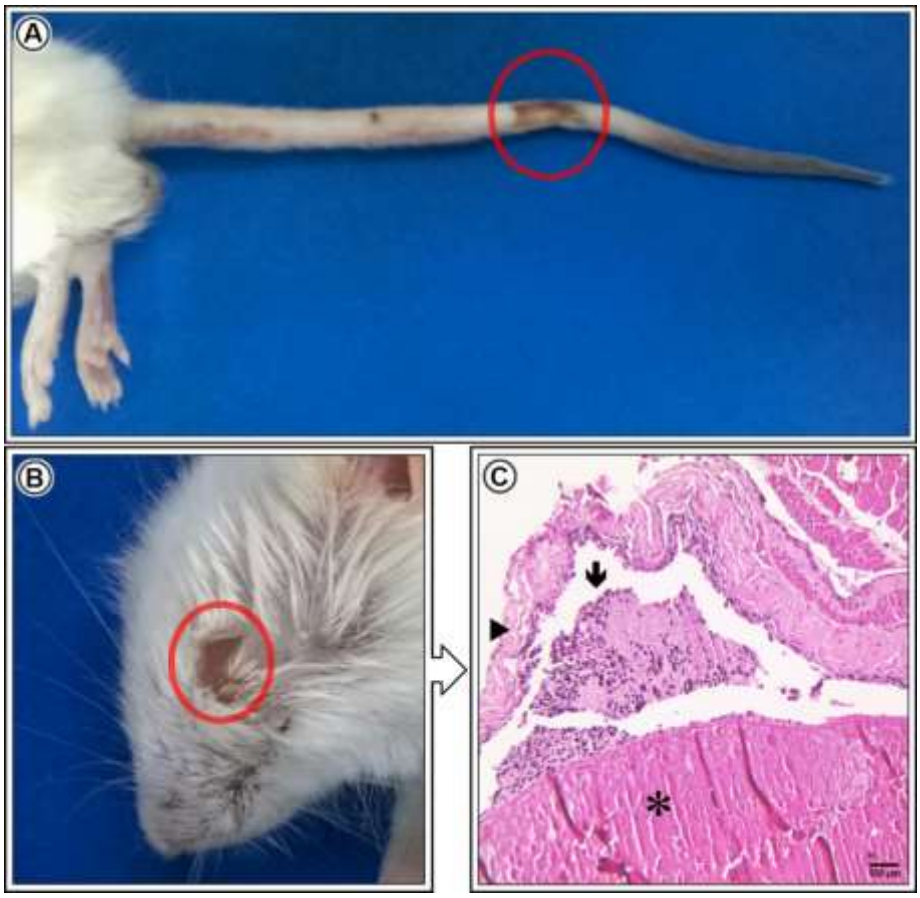

Figure 2. Tail and eye infection. (A) Tail of mouse inoculated with MRSA *pvl* (+) presenting ulceration and hyperemia, distal region with necrosis and region near the base with edema. (B) Inflamed eye with palpebral edema presenting thick yellowish material lining the sclera. (C) Photomicrography (100x), eyeball (asterisk) stained with H&E, presenting inflammatory exudate composed of polymorphonuclear leukocytes and fibrin (arrow), epithelium with necrosis region and leukocyte invasion (arrowhead).

local inoculation route (Diep and Otto, 2008). The fact that strains producing PVL are capable of causing dermonecrosis in rabbits and mice reinforces the hypothesis of a selective advantage in MRSA *pvl* (+) among healthy individuals. This report demonstrates the great importance in the sterilization of the hospital environment and surgical materials for the reduction of the dissemination of this microorganism, avoiding serious infections in hospitalized patients, because invasive surgical procedures and open cutaneous lesions are access doors for *S. aureus* to invade healthy tissues and through the bloodstream causes bacteremia and colonizes vital organs, leading to sepsis and death (Boyle-Vavra and Daum, 2007; Ward and Turner, 1980).

Infection models using animals can only partly reproduce the effect of pathogens on development of diseases in humans (Diep and Otto, 2008). Studies using PVL-purified in intravenous inoculation models demonstrated null effect in rats and rabbits, leading to the belief that action of PVL is associated with specific interactions with human neutrophils (Diep and Otto, 2008). However, in studies reported above it was possible to observe high intensity infections caused by strains producing PVL in Swiss mice having as an inoculation pathway, the vascular system.

The interactions between pathogen-host have not yet been well elucidated, mainly because MRSA strains do not secrete only PVL, as well as other exotoxins with leucolytic activities (Diep and Otto, 2008). In mice, intravenous inoculation with *S. aureus* triggers dissemination through the blood to other tissues and organs, where they establish lesions and abscess in skeletal muscle, in the vasculature, brain, lungs, heart, liver, spleen and kidneys (Cheng et al., 2009; Kim et al., 2014). In this study, *S. aureus* was able to cause intense local infection in skin and soft tissues, however, PVL-producing strains were more apt to migrate through the blood and cause infection in organs distant from inoculation site, as the eyes. This result reinforces the idea of PVL targeting polymorphonuclear leukocytes and

macrophages that are the first defense barriers of immune system, causing cytolysis and apoptosis in these cells, enabling the evasion of the immune system (Genestier et al., 2005; Boyle-Vavra and Daum, 2007).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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