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Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Changes of nucleotide-binding oligomerization domains (NODs) signaling pathway in the incidence and development of invasive pulmonary aspergillosis

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This study investigates the effect of nucleotide-binding oligomerization domains (NODs) signal pathway in invasive pulmonary aspergillosis. Mice were randomly divided into three groups: 1) normal mice (control group), 2) normal mice infected with *Aspergillus fumigates,* 3) normal mice treated with immunosuppressant and inoculated with *A. fumigatus* (IPA Model). Mice were sacrificed at different time points after inhaling *A. fumigatus* spores by nose. Their lungs were extracted under sterile condition, and were used to count the fungal colonies; and also the pathological sections of lungs were observed by HE staining. RT-PCR was used to detect the expression of the NOD1, NOD2 and RIP2 mRNA of mice lung. Western blot was used to detect the expression of TNF-α. 72 h after inhaling *A. fumigatus* spores, a large number of hyphae and severe inflammation were found in the lung of IPA model mice group; and the lung burden of IPA mice were more than that of normal+*A. fumigatus* group at each time points. When compared with normal+*A. fumigatus* group, the expressions of NOD1 and RIP2 mRNA were persistently descending in IPA model mice group; the expression of NOD2 mRNA was abnormally raised in early stage of infection (24 h), then decreased in the later stage. However, in normal+*A. fumigatus* group, proinflammatory cytokine TNF-α exhibited high expression at the early stages of infection, and the highest expression levels appeared at 48 or 72 h, then decreased and returned to normal level. In the group of the IPA mouse, proinflammatory cytokines TNF-α were released at slow and low level. Persistently low expression of NOD1 and RIP2, was seen in early excessive activation.

Key words: Invasive pulmonary aspergillosis (IPA), nucleotide-binding oligomerization domains (NODs), RIP2, pathogenesis.

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is an increasingly common opportunistic fungal infection usually occurring in patients with neutropenia and/or corticosteroid exposure. The lungs are involved in about 85% of cases

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of invasive aspergillosis. *Aspergillus fumigatus* is a common saprophytic fungus in the air. It has a small diameter and can be passively inhaled into the respiretory tract. *A. fumigatus* conidia in hosts with impaired immunitycan cause a severe infectious disease called IPA. It is also responsible for some autoimmune diseases, including rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, psoriasis and psoriatic arthritis (Arnold et al., 2009; Tsiodras et al., 2008; Nedel et al., 2009). The mortality rate of IPA has ranged from 60 to 94% (Tomee, 2001; Singh and Paterson, 2005). Hitherto, the pathogenesis of IPA is not clear.

Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are two major pattern-recognition receptors (PRRs) involved in the early host defense against pathogen invasion (Akira et al., 2006; Li, 2010). Activation of NOD1 by *A. fumigatus* conidia ligand recognition elicited inflammatory cytokines such as TNF-α and IL-10 and stimulated NOD1-induced immune responses (Li, 2010). NOD2 can sense the components of peptidoglycan derived from bacteria, such as muramyldipeptide (MDP), in the host cytosol. Stimulation of NOD2 by ligand recognition stimulates the NF-kB pathway(Chattoraj et al., 2013; Hasegawa et al., 2008; Nabatov et al., 2013). All these studies showed that NODs play important roles in anti-infectious disease IPA. But there are few studies on the field.

Therefore, we established IPA model of wild type mouse and dynamic investigation of the expression levels of NOD1 and NOD2 mRNA by RT-PCR method, the levels of inflammatory cytokines TNF-αin pulmonary tissues by western blot, with evaluation of the *A. fumigates* dosage, and the lung pathology; we elucidated the functions of NODs signaling pathway in invasive pulmonary aspergillosis. This study provides an insight into the pathogenesis of IPA.

MATERIALS AND METHODS

Experimental animals and grouping

BALB/cSPF mice (Certificate of Conformity: SCXK 2003-0002, male, 6 to 8 weeks old, 20~25 g) were provided by Shanghai SINO-BRITISH SIPPR / BKLAB animal center. Mice were divided into 3 groups randomly, 10 rats in each group: (1)Normal Group (normal mice); (2) Normal mice with infection (N+*A. fumigates*); (3) IPA Model Group (normal mice treated with immunosuppressant and inoculated with *A. fumigates*).

Strain and culture medium

A. fumigates (clinical isolates, Separate No. 3910): was purchased from the Fungal Culture Collection of Chinese Medicine Centre (Nanjing). Cells were cultured in Czapek's medium at 26°C. Spores were collected at the concentration of 10^7/mL and stored at 4°C.

Main reagents

Cyclophosphamide (CY,NO.:06060521) was purchased from Jiangsu Hengrui Medicine Co., Ltd.; Trizol reagent was from invitrogen company; TaKaRa RNA PCR Kit 3.0(AMV) was purchased from Dalian TaKaRa Biotechnology Co., Ltd.; antibodies (Rabbit anti-NF-κB p65, Rabbit anti-IL-1β, Goat anti-rabbit HRP secondary antibodies) were purchased from Santa Cruz Biotechnology (Beijing, China).; PCR primers were designed in our lab and from Shanghai Biological Engineering company; ultrapure water (UPW,NO.:07020201) was from U.S. MIUIOORE Inc.

IPA model of mice

According to the literature, the method was as follows: BALB/c mice were injected intraperitoneally with 100 $mg \cdot kg^{-1} \cdot d^{-1}$ of CY within 2 days. Thereafter, mice were administered intranasally with 50 μL (concentration: 10^7/mL) spore suspension of *A. fumigates*. In order to maintain the effect of immunosuppression, mice were given additional CY (100 $mg \cdot kg^{-1} \cdot d^{-1}$) when inoculated with *A. fumigates* at 96h (Luo et al., 2008; Tang et al., 1993).

Collection and processing of specimen

Mice with nose inhalation of *A. fumigates* conidia were sacrificed at different time points of 24, 48, 72, 120 and 144 h (2 mice at each time point), then lung tissue was isolated in sterile manner, and conserved in -80°C refrigerator.

A. fumigates colony counting of lung tissue

100 mg of lung tissue were took and made into 10% homogenate, then 0.1 ml of it was inoculated on Czapek's medium after diluting 100 times, and counting colony after 5 days.

Lung tissue pathology

Histological injury and spore germination was observed after all the mice produced paraffin sections of lung tissue and conventional HE staining.

Detection of the target genes expression of lung tissue by RT-PCR

Primers and PCR reaction conditions are listed in Table 1.

Detection of the target proteins expression of lung tissue by Western blot

First, nuclear protein and total protein were extracted from 100 mg lung tissue; Second, SDS-PAGE electrophoresis was carried out and transferred to semi-dry membrane; once again, it was incubated with the corresponding primary antibody (1:250) and secondary antibodies (1:8000) at 37°C for 1 h; finally, the film was exposed to X-ray after colouring with ECL (a kind of lighting substrate used in west blot detection, Pierce Biotechnology PO Box).

Statistical methods

The values of optical density scanning of target band were read which was measured in the agarose gel and X-ray film by image analysis software Bandscan. Afterwards, the expression of its corresponding target gene and protein were respectively

Table 1. Primer sequences and PCR reactive conditions.

Aim gene	Primers (5′>3′)		Annealing temperature (°C)	Length (bp)
β-Actin	reverse anti-reverse	ACGGCCAGGTCATCACTATT TAACAGTCCGCCTAGAAGCA	59.3	409
NOD1	reverse anti-reverse	CAACAGGGAACATCTGGTCA GAAGGGGAGAAGCCAATTTC	67.7	261
NOD2	reverse anti-reverse	CCGTGTCCTGTTAACCTTTG AGGATCAGCAGGTACATGTC	59.3	438
RIP2	reverse anti-reverse	GCCATTGTGAGCCAGATGA ATTTGAAGGCGGTGCTTTG	59.3	264

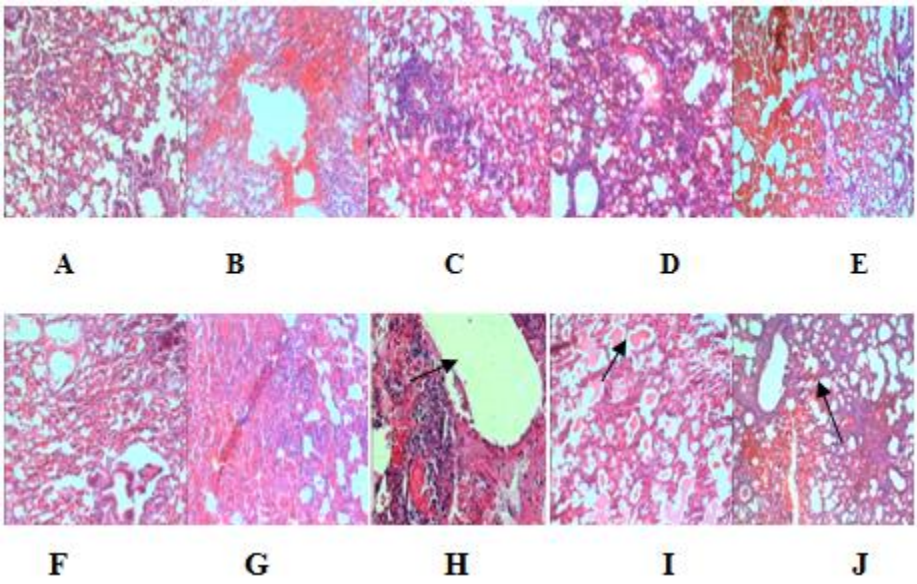

Figure 1. Results of HE staining of the lungs. A−E: N+*A. fumigates* group: 24, 48, 72, 120, 144 h; F−J: IPA group: 24, 48, 72, 120, 144 h.

standardized by scanning values in each group of β-tublin and β-actin bands. Here, each experiment was repeated three times, and results were indicated as $\overline{X} \pm S$. P<0.05 was used as a standard of significant difference by applying Statistical software SPSS 10.0 to conduct t-test analysis.

RESULTS

Morphological analysis of lung pathology

There were few lung abscess, hemorrhage and mycelium in lung tissue in the first 48 h after inoculation with *A. fumigates*; 72 h later, when compared with normal mice, the alveolus space in normal mice with infection enlarged, accompanied with inflammatory responses including inflammatory cell infiltration and hemorrhage injury. As a comparison, IPA group had a lung abscess and severe hemorrhage. In addition, airway epithelial desquamation and mycelium formation were also observed in the IPA group (Figure 1).

Assessment of *A. fumigates* load in pulmonary tissue

The CFU assay indicated, when compared with normal mice with infection, pulmonary tissue from the IPA group had heavy *A. fumigates* load (P<0.05) (Figure 2). In contrast, normal mice without *A. fumigates* inoculation showed negative signal in this assay.

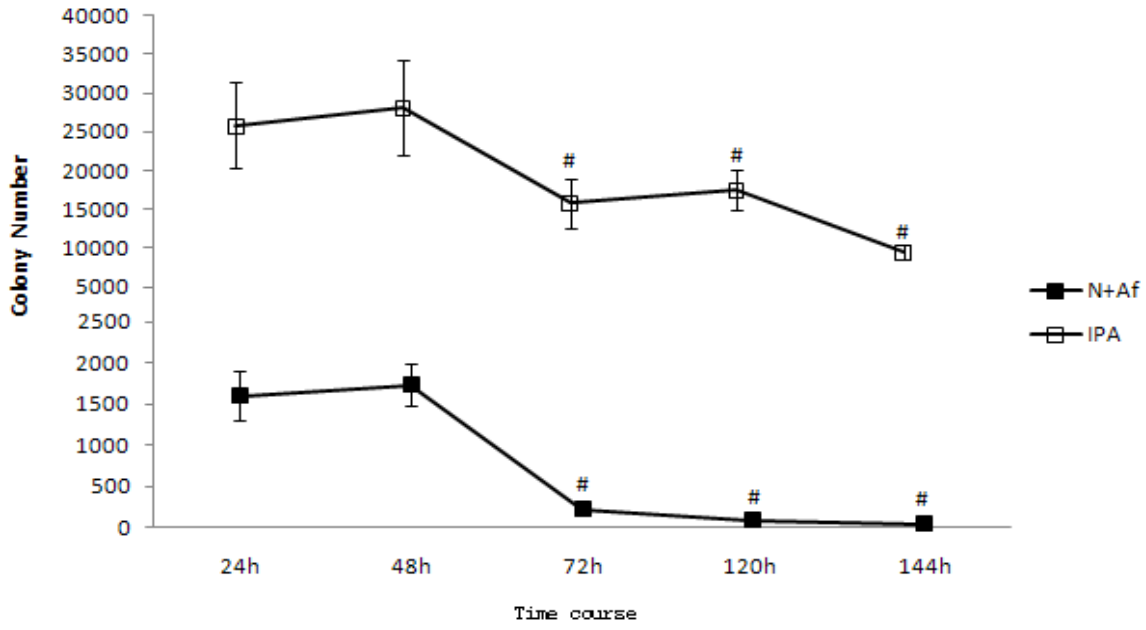

Figure 2. The trend of *A. fumigatus* load in lung tissue of normal mice with infection group and IPA group. N+*Af*: N+*A. fumigates* group; IPA: IPA group; #*P*<0.05, vs at 24 h.

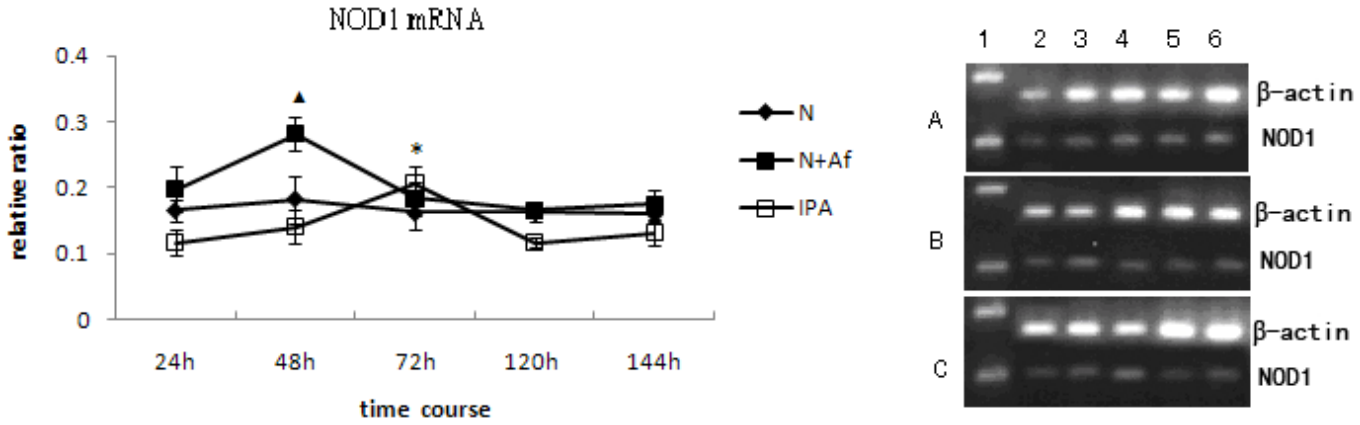

Figure 3. The dynamic expression of NOD1 mRNA in different group and the product's electropherogram of NOD1 gene at different time point. N: Normal group; N+*Af*: N+*A.fumigates* group; IPA: IPA group. A: Normal group; B: N +*A.fumigates* group; C: IPA group. 1: Marker; 2: 24 h; 3: 48 h; 4: 72 h; 5: 120 h; 6: 144 h. ▲*P*<0.05, vs at 24 h in N+*A. fumigates* group; **P*<0.05, vs at 24 h in IPA group.

Investigating the expression levels of NOD1 mRNA in mice pulmonary tissue

The mRNA levels of NOD1 were tested with RT-PCRby time course (Figure 3). Twenty-four hours after inoculation with *A. fumigates,* the expression of NOD1 protein of normal group (N+*Af*) had a sharp elevation, got to peak at 48 h and then decreased to normal levels after 72 h. IPA mice of NOD1 protein gradually increased after 24 h. It peaked at 72 h, followed by a decline in a level lower than normal mice with infection (*P*<0.05) (Figure 3).

Investigating the expression levels of NOD2 mRNA in mice pulmonary tissue

The mRNA levels of NOD2 were tested with RT-PCRby time course (Figure 4). Twenty-four hours after inoculation with *A. fumigates,* the expression of NOD2 protein of normal group (N+*Af*) had a sharp elevation, got to peak at 48 h and had a little decline, and then kept to high levels after 72 h. IPA mice of NOD2 protein gradually increased after 24 h. It peaked at 48 h, followed by a slowly decline, and finally at 120 h, got back to normal

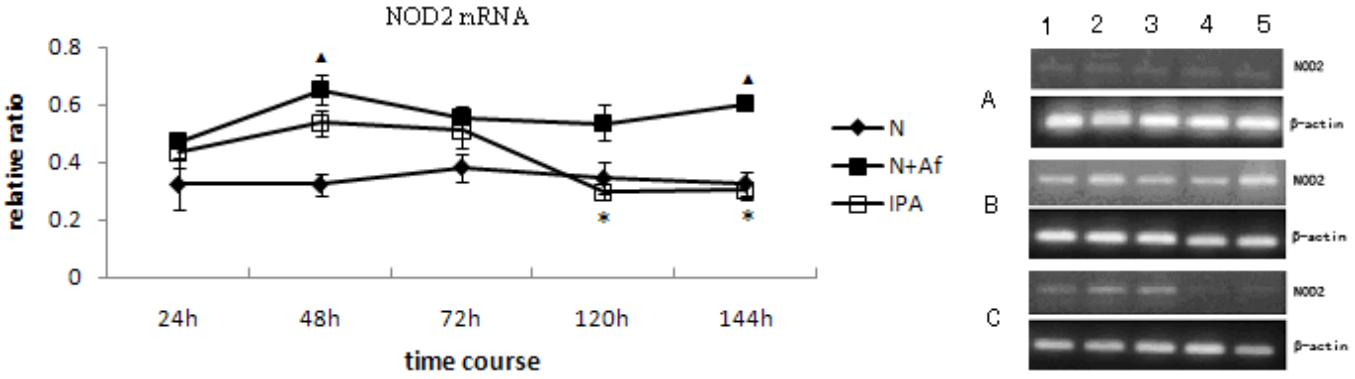

Figure 4. The dynamic expression of NOD2 mRNA in different group and the product's electropherogram of NOD2 gene at different time point. N: Normal group; N+*Af:* N+*A. fumigates* group; IPA: IPA group. A: Normal group; B: N +*A.fumigates* group; C: IPA group.1: 24 h; 2: 48 h; 3: 72 h; 4: 120 h; 5: 144 h. ▲*P*<0.05, vs at 24 h in N+ *A. fumigates* group; **P*<0.05, vs at 24 h in IPA group.

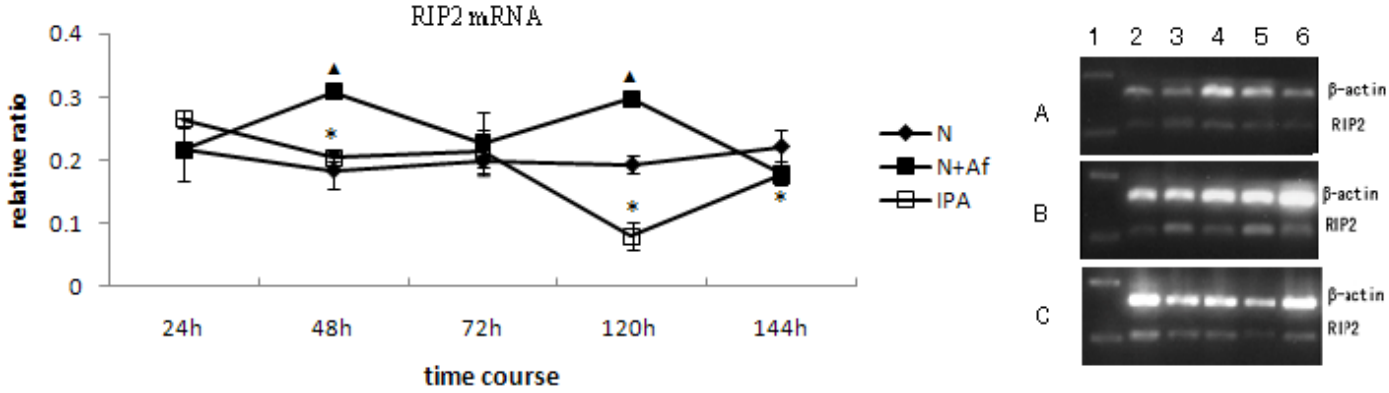

Figure 5. The dynamic expression of RIP2mRNA in different group and the product's electropherogram of RIP2gene at different time point. N: Normal group; N+*Af :* N+*A.fumigates* group ; IPA: IPA group. A: Normal group; B: N +*A. fumigates* group; C: IPA group.1: Marker; 2: 24 h; 3: 48 h; 4: 72 h; 5: 120 h; 6: 144 h. ▲*P*<0.05, vs at 24 h in N+ *A. fumigates* group; **P*<0.05, vs at 24 h in IPA group.

level (*P*<0.05) (Figure 4).

Investigating the expression levels of RIP2 mRNA in mice pulmonary tissue

The mRNA levels of RIP2 were tested with RT-PCRby time course (Figure 5). Twenty-four hours after inoculation with *A. fumigates,* the expression of RIP2 protein of normal grou p(N+*Af*) had a sharp elevation, got to peak at 48 h. Then had a quick decline, and then got back to normal levels at 72 h; it had another peak at 120 h; and then decline to normal level. IPA mice of RIP2 protein gradually decreased after 24 h; and got to lowest point at 120 h, followed by a sharp increase, and finally got back to normal level (*P*<0.05) (Figure 5).

Analyzing the expression of TNF-α protein

Time course experiments were conducted to evaluate the expression of TNF-α protein in mouse pulmonary tissues from different treatment groups. As shown in Figure 6, TNF-α protein levels were measured by western blot.

Twenty-four hours after inoculation with *A. fumigates,* the expression of TNF-αprotein of normal group (N+*Af*) gradually increased, and then got to the peak at 72h; then gradually decline to normal level at 144 h. As a comparison, IPA mice had a lower TNF-α expression with mild alternation (Figure 6).

Analyzing the expression levels of NOD1, NOD2, RIP2 mRNA and the expression of TNF-α at the same time point for each group

The mRNA levels of NOD1, NOD2 andRIP2 were compared at the same time point for each group. The expression of NOD1 protein of normal group (N+*Af*) are higher than normal group at each time course (*P*<0.05). The expression of NOD2 protein of normal group (N+*Af*)

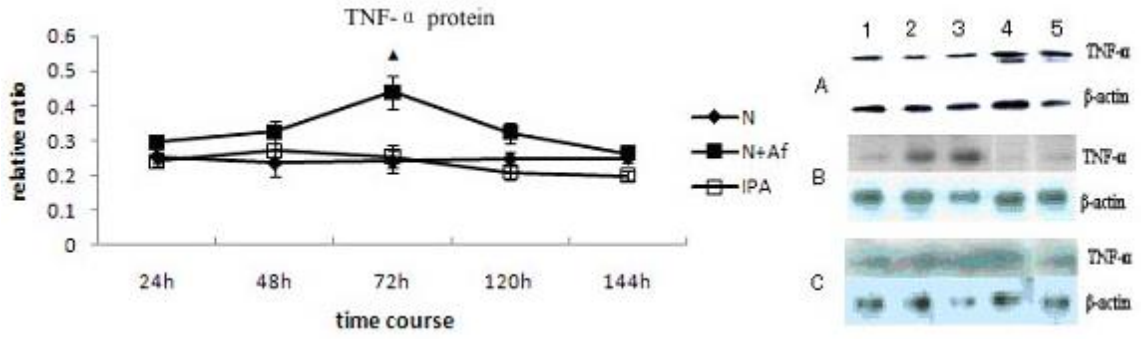

Figure 6. The dynamic expression of TNF-α protein in different group and the western blotting gram of TNF-α protein at different time point. N: Normal group; N+*Af :* N+*A.fumigates* group; IPA: IPA group. A: Normal group; B: N +*A.fumigates* group; C: IPA group.1: 24 h; 2: 48 h; 3: 72 h; 4: 120 h; 5: 144 h. ▲*P*<0.05, vs at 24 h in N+ *A. fumigates* group; * *P*<0.05, vs at 24 h in IPA group.

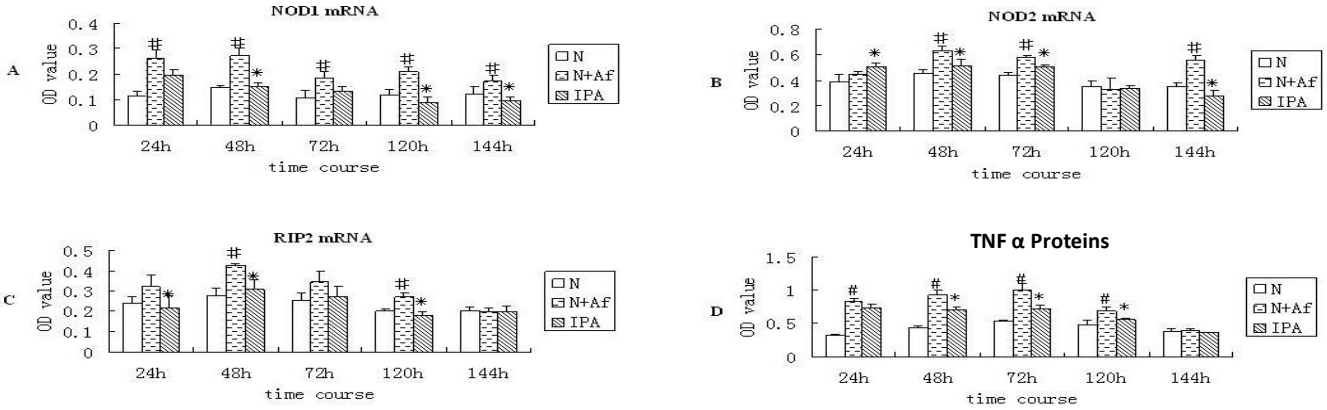

Figure 7. The expression of NODs and RIP2 mRNA and TNF-α protein at the same time in different groups. N: Normal group; N+*Af*: N+*A. fumigates* group; IPA: IPA group. #*P*<0.05, vs N group; **P*<0.05, vs N+ *A. fumigates* group.

are more than normal groups at 48, 72 and 144 h(*P*<0.05). RIP2 is higher than normal group at 48 and 72 h after incubation with *A. fumigates* (*P*<0.05). When compared with normal group, the expression of NOD1 protein of IPA group is lower at 48, 120 and 144 h (*P*<0.05); NOD2 are less at all the time point; RIP2 are lower at 24, 48 and 120 h (*P*<0.05) (Figure 7).

The expression of TNF-α protein in mouse pulmonary tissues was also compared at the same time point for each group. When compared with normal group, TNF-α protein of normal group (N+*Af*) is higher at all the time point except at 144 h (*P*<0.05); TNF-α protein of IPA group is lower at 48, 72 and 120 h (*P*<0.05) (Figure 7D).

DISCUSSION

Since conidia of *A. fumigates* widely exist in the environment, people usually inhale hundreds of them per day. The inspiratory conidia seldom induce disease in hosts with intact immune system. High risk factors for clinical IPA usually are due to severe neutropenia, long-term antibiotic treatment, steroid therapy, hematopoietic malignancies, organ transplantation, AIDS, autoimmune diseases, etc. Animal experiments showed: after high dose inoculation with *A. fumigates* in normal mice, the pathogens would be eliminated within several hours, and the elimination curve was in accordance with first order kinetics; whereas immuno-deficient mice easily suffered from aspergillosis and IPA induced by systemic infection (Schneemann and Schaffner, 1999; Grazziutti et al., 1997; Duong et al., 1998). Thus, healthiness of immune system plays pivotal roles in resistance to *A. fumigates* infection.

Effective innate immunity is the first line of defense. Phagocytosis of the alveolar macrophages kills inhalational conidia and prevents the formation of hyphae, which can colonize in the host and are associated with lethal infection. Once the conidia escape from phagocytosis and develop into hyphae, neutrophils

will take over the defense line. At the same time, macrophages and lung dendritic cells phagocytize conidia and hyphae, present antigens and initiate T cell immune response. Innate immunity not only confers the first line of defense in resistance to *A. fumigates* infection, but also provides specific signals for initiation of adaptive immunity (de Repentigny et al., 1993; Schaffner et al., 1982). However, the mechanism of innate immunity against the infection of *A. fumigates* is still largely unknown.

To activate host defense and eliminate invasive pathogens, innate immune response is initiated by pattern recognition,a conserved and pathogen-specific molecular recognition pattern mediated by a series of PRRs that widely express in macrophages and various cell types (Medzhitov and Janeway, 1997a, b). The NODs family, which was newly discovered, is one of the most important PRRs (Philpott and Girardin, 2004). Most evidence proved that NOD1 and NOD2 play important role in anti-infectious in innate immune response (Boughan et al., 2006; Kobayashi et al., 2005; Chamaillard et al., 2004; Barton et al., 2007). Innate immunity not only confers the first line of defense in resistance to *A. fumigates* infection, but also provides specific signals for initiation of adaptive immunity (Mambula et al., 2002; Kaisho and Akira, 2001). However, the mechanism of innate immunity against the infection of *A. fumigates* is still largely unknown.

Here, in order to systematically mimic patient IPA, the dynamic alternations of NODs, PIR2 and TNF-αcytokines in both normal and immunodeficient mice were evaluated during infection of *A. fumigates*. Cyclophosphamide was used to induce immunosuppression of mice used as model animals. And, the pathological alternation of pulmonary tissues and culture of *A. fumigates* in normal mice with those in immunosuppressive mice after nose inhalation of *A. fumigates* conidia were compared.

Lung pathology analysis showed that normal mice infected with *A. fumigates* displayed obvious inflammatory responses in the early stage (24 to 48 h) of infection; hyperemia and hemorrhage gradually declined at 72 h, and nearly disappeared at 144 h. IPA group had mild inflammatory responses in the early stage of infection. The severe hyperemia and hemorrhage occurred after 72 h. At the same time, lung abscess and enlargement of alveolus space, together with airway epithelial desquamation and mycelium formation, were observed. After 144 h, mesothelial hyperplasia became remarkable in the lung tissues of IPA mice.

Data from analysis of *A. fumigates* load in pulmonary tissues showed that normal infected mouse lung displayed a positive signal in CFU assay between 24 and 48 h, but the signal reduced dramatically after 48 h; whereas positive signal was detected at 24 h in IPA group, and the strong signal could be detected even at 144 h. The number of colonies in IPA group was higher than those in normal mice with infection at all-time points ($P<0.05$).

The above results showed immunosuppressed mice with nose inhalation of *A. fumigates* presented pathological alternations similar to clinical IPA cases, indicating the successful establishment of mouse IPA model. Analysis of pulmonary histology combined with CFU assay reminded us that immunosuppressive mice were not able to effectively initiate immune responses, which caused late inflammatory reactions in the early stage for elimination of conidia and suppression of hyphae growth. On the contrary, overreacted inflammatory responses in the late stage of infection led to severe damage of lung tissues.

NODs signaling is the important network for the regulation of inflammatory and immune response, and also the major pathway for resistance to infection. In this study, we discovered the different dynamic expression pattern of NOD1 and NOD2 mRNA between IPA group and normal mice with infection. NODs and RIP2 mRNA in the three groups was slowly increased in the early stage of *A. fumigates* infection. These results indicated that the receptors of NODs and RIP2 were activated after *A. fumigates* inoculation. The expression of NOD2 mRNA in IPA group was higher than normal group in early stage, and then got back to normal level; this indicated that NOD2 may play very import role in *A. fumigates* infection.

Cytokines, an important kind of secretive immune molecules, play roles in diverse biological functions including regulation of cell physiology, mediation of inflammatory responses, involvement of immune reactions, and repair of tissues, etc. The different functions of various cytokines are closely related with the situation and progression of infectiousdiseases (Peck and Mellins, 2010).

We found the expression levels of cytokines (TNF-α) in mouse lung were closely correlated with pulmonary pathological impairment. Normal mice with *A. fumigates* inoculation displayed high levels of proinflammatory cytokines (TNF-α) in the early stage of infection. Their expression levels peaked at 72 h, and thereafter declined to normal level. At the same time, lung pathology results showed obvious hyperemia and hemorrhage appeareance before 72 h, and thereafter inflammatory responses were gradually alleviated, which indicated that secretion of proinflammatory cytokines (killing inhalational conidia and preventing the formation of hyphae) was the major inflammatory responses in the early stage of *A. fumigates* infection,

Inflammation is one of the necessary parts of effective immune responses in resistance to IPA. Appropriate inflammatory responses can availably eliminate local *A. fumigates*, whereas improper or overreacted inflammatory responses will cause IPA and associated lung injury (Romani and Puccetti, 2007). Effective inflammatory responses depend on the mutual cooperation or restriction between diverse immunocytes, which ultimately help the host eliminate exotic antigens

as well as protect its own tissues by the regulation of the secretions and functions of diverse cytokines. Recognition of pathogens by PRRs is the key to innate and adaptive immunities. Multiple regulations ensure complicate but appropriate activation of signaling pathways. Abnormal activation of upstream and midstream molecules in signaling pathways will affect their downstream networks, and finally cause inflammatory diseases (Medvedev et al., 2006).

The results indicates that the NODs signaling pathway in the immunosuppressed mice with *A. fumigates* inoculation causes the loss of balance between proinflammatory and anti-inflammatory cytokines and eventually leads to the incidence and development of invasive aspergillosis.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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REFERENCES

Akira S, Uematsu S, Takeuchi O (2006). Pathogen recognition and innate immunity. Cell 124(4):783-801.

Arnold TM, Sears CR, Hage CA (2009). Invasive fungal infections in the era of biologics. Clin. Chest Med. 30(2):279-286.

Barton JL, Berg T, Didon L, Nord M (2007). The pattern recognition receptor Nod1 activates CCAAT/enhancer binding protein beta signalling in lung epithelial cells. Eur. Respir. J. 30(2):214-222.

Boughan PK, Argent RH, Body-Malapel M, Park JH, Ewings KE, Bowie AG, Ong SJ, Cook SJ (2006). Sorensen OE, Manzo BA *et al*: Nucleotide-binding oligomerization domain-1 and epidermal growth factor receptor: critical regulators of beta-defensins during Helicobacter pylori infection. J. Biol. Chem. 281(17):11637-11648.

Chamaillard M, Inohara N, Nunez G (2004). Battling enteroinvasive bacteria: Nod1 comes to the rescue. Trends Microbiol. 12(12):529-532.

Chattoraj P, Yang Q, Khandai A, Al-Hendy O, Ismail N(2013). TLR2 and Nod2 mediate resistance or susceptibility to fatal intracellular Ehrlichia infection in murine models of ehrlichiosis. PLoS One 8(3):e58514.

de Repentigny L, Petitbois S, Boushira M, Michaliszyn E, Senechal S, Gendron N, Montplaisir S(1993). Acquired immunity in experimental murine aspergillosis is mediated by macrophages. Infect. Immun. 61(9):3791-3802.

Duong M, Ouellet N, Simard M, Bergeron Y, Olivier M, Bergeron MG (1998). Kinetic study of host defense and inflammatory response to Aspergillus fumigatus in steroid-induced immunosuppressed mice. J. Infect. Dis. 178(5):1472-1482.

Grazziutti ML, Rex JH, Cowart RE, Anaissie EJ, Ford A, Savary CA (1997). Aspergillus fumigatus conidia induce a Th1-type cytokine response. J. Infect. Dis. 176(6):1579-1583.

Hasegawa M, Fujimoto Y, Lucas PC, Nakano H, Fukase K, Nunez G, Inohara N (2008). A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. Embo J. 27(2):373-383.

Kaisho T, Akira S (2001). Toll-like receptors and their signaling mechanism in innate immunity. Acta Odontol. Scand. 59(3):124-130.

Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, Flavell RA (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science 307(5710):731-734.

Li X, Luo H-D, Shi Q (2010). The activation of TLRs/NF-κB signal pathways in the invasive pulmonary as- pergillosis of mice. Chinese J. Immunol. 26(10):881-886.

Luo HD, Liu JH, Dai DD, Long K, Xie XM (2008). The expression of TLR2 and TLR4 in invasive pulmonary aspergillosis mice. Chin. J. Microbiol. Immunol. 28(8):685-689.

Mambula SS, Sau K, Henneke P, Golenbock DT, Levitz SM (2002).Toll-like receptor (TLR) signaling in response to Aspergillus fumigatus. J. Biol. Chem. 277(42):39320-39326.

Medvedev AE, Sabroe I, Hasday JD, Vogel SN (2006). Tolerance to microbial TLR ligands: molecular mechanisms and relevance to disease. J. Endotoxin. Res. 12(3):133-150.

Medzhitov R, Janeway CJ (1997a). Innate immunity: impact on the adaptive immune response. Curr. Opin. Immunol. 9(1):4-9.

Medzhitov R, Janeway CJ (1997b). Innate immunity: the virtues of a nonclonal system of recognition. Cell 91(3):295-298.

Nabatov AA, Hatzis P, Rouschop KM, van Diest P, Vooijs M (2013). Hypoxia inducible NOD2 interacts with 3-O-sulfogalactoceramide and regulates vesicular homeostasis. Biochim. Biophys. Acta 1830(11):5277-5286.

Nedel WL, Kontoyiannis DP, Pasqualotto AC (2009). Aspergillosis in patients treated with monoclonal antibodies. Rev. Iberoam. Micol. 26(3):175-183.

Peck A, Mellins ED (2010). Precarious balance: Th17 cells in host defense. Infect. Immun. 78(1):32-38.

Philpott DJ, Girardin SE (2004). The role of Toll-like receptors and Nod proteins in bacterial infection. Mol. Immunol. 41(11):1099-1108.

Romani L, Puccetti P (2007). Controlling pathogenic inflammation to fungi. Expert Rev. Anti Infect. Ther. 5(6):1007-1017.

Schaffner A, Douglas H, Braude A (1982). Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to Aspergillus. Observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes. J. Clin. Invest. 69(3):617-631.

Schneemann M, Schaffner A (1999). Host defense mechanism in Aspergillus fumigatus infections. Contrib. Microbiol. 2:57-68.

Singh N, Paterson DL (2005). Aspergillus infections in transplant recipients. Clin. Microbiol. Rev. 18(1):44-69.

Tang CM, Cohen J, Krausz T, Van Noorden S, Holden DW (1993). The alkaline protease of Aspergillus fumigatus is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect. Immun. 61(5):1650-1656.

Tomee JF VDWT (2001). Pulmonary aspergillosis. Neth. J. Med. 244-258.

Tsiodras S, Samonis G, Boumpas DT, Kontoyiannis DP (2008). Fungal infections complicating tumor necrosis factor alpha blockade therapy. Mayo Clin. Proc. 83(2):181-194.

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Challenges in predicting *Staphylococcus* spp. β-lactamic resistance in pet animals

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The present work evaluated the species distribution associated with the antimicrobial susceptibility pattern by phenotypic analysis and *mec* and *bla* gene detection in 100 *Staphylococcus* strains from 219 clinical samples from cats and dogs in the state of Rio de Janeiro, Brazil. Oxacillin susceptibility profiles were detected by the Clinical and Laboratory Standards Institute (CLSI) recommended tests. The amplification of the *mecA* gene was positive in 25% of the *Staphylococcus* spp. The whole *mec* complex (*mecA-mecI-mecR1*) was detected in four phenotypically oxacillin-resistant isolates. The CLSI recommended nitrocefin-based test detected 38% (38/100) β-lactamase producers *Staphylococcus* strains. Also, 32% (32/100) *Staphylococcus* spp. strains tested positive for *bla* genes. The whole *bla* gene complex, *blaZ-blaI-blaR1*, was detected in 7.8% (3/38) of the nitrocefinase-positive isolates. β-Lactamases was well spread among the samples and it seems to be a prevalent mechanism in resistant staphylococci strains from pet animals. The *mec* and *bla* gene regulatory systems can interfere in expression of resistance mediated by PBPs and β-lactamases, conferring the heterogeneous oxacillin-resistance in *Staphylococcus* spp. detected by phenotypic tests.

Key words: *Staphylococcus* spp., oxacillin, β-lactamase, *mec*A, pet animals.

INTRODUCTION

In veterinary medicine, *Staphylococcus* spp. are important agents of infectious diseases of several animal species. Besides *Staphylococcus aureus*, other coagulase-positive staphylococci (CoPS) have been reported to be important pathogens. The reclassification of *Staphylococcus intermedius,* a coagulase-positive species firstly described in 1976, was proposed by Devriese et al. (2005), creating the *S. intermedius* group (SIG) including *S. intermedius,* the new species *S. pseudintermedius* and *S. delphini*. This proposal was based in the high genotypic diversity observed in the formerly considered *S. intermedius* strains (Bannoehr and Guardabassi, 2012).

Like *S. aureus*, the *S. intermedius* strains isolated from

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animals have been reported to produce an array of virulence factors, including leukotoxin, enterotoxin, and hemolysins, together with elements essential for biofilm formation (Hanselmann et al., 2008). Nowadays, several CoPS species, such as *S. aureus*, *S. pseudintermedius, S. schleiferi* subsp. *coagulans,* and also coagulase-negative *Staphylococcus* spp. (CoNS), are implicated in the etiology of animal diseases, such as suppurative disease, mastitis, arthritis and urinary tract infection, due to their virulence factors (Futagawa-Saito et al., 2006; Silva et al., 2003). *Staphylococcus* species are usually resistant to ß-lactams, aminoglycosides and macrolides. The emergence and dissemination of antimicrobial resistance among staphylococci is an important problem in human and veterinary medicine worldwide because therapeutic options are becoming limited. Dogs and cats are an important source of the spread of this resistance due to the extensive use of antimicrobials and the contact with their owners (Frank and Loeffler, 2012; McCarthy et al., 2012; Morgan, 2008).

Oxacillin resistance is of particular relevance because it is conferred by different mechanisms and acts as a resistance marker for overall beta-lactamic resistance. The most-studied mechanism is related to the presence of the *mecA* gene, located on a staphylococcal chromosomal cassette (SCCmec) (Ito et al., 2001). Jansen et al. (2009) sequenced and typed SCCmec of animal origin (Jansen et al., 2009). This resistance is conferred by the production of an altered penicillin binding protein (PBP2a) with low affinity for all β-lactamic antimicrobials. The *mecA* gene expression and therefore PBP2a production is regulated by the *mecR1-mecI* gene system. The *mecI* gene codifies a repressive protein and *mecR1* a signal transmembrane protein inducible by β-lactamic antimicrobials (Petinaki et al, 2001). Whether these genes are expressed or not, they can confer heterogeneous phenotypes, possibly leading to misidentification by laboratory practitioners. Usually the SCCmec contains additional genetic material, such as *Tn554*, pUB110 and pT181, which encode resistance to multiple classes of antimicrobials frequently applied in hospitals (Hanselmann et al., 2008; Katayama et al., 2001).

Also, oxacillin-resistant *Staphylococcus* may constitute or inductively produce ß-lactamases enzymes that cleave the ß-lactam ring and inactivate the antibiotic (Li et al., 2007). ß-lactamase enzymes interfere in oxacillin resistance by the action of the *blaZ* gene complex, which includes a regulatory system composed of *blaZ*, *blaR1* and *blaI* genes (Rosato et al., 2003). These genes are located on Tn552, a transposon completely sequenced and inserted in SCC*mec* (Rowland and Dyke, 1990). More than 90% of staphylococcal isolates that produce ß-lactamase codified by the *blaZ* gene contain a *blaZ* regulatory system (*blaI* and *blaR1*) similar in sequence and function to *mecA* regulators (*mecA-mecR1-mecI*, promoter-operator-repressor system) (Mckinney et al., 2001).

The Clinical and Laboratory Standards Institute (CLSI) has standardized phenotypic testing for detection of oxacillin resistance considering the use of the cefoxitin/oxacillin disk diffusion test according to *Staphylococcus* species. In its latest version (CLSI, 2013), *mecA* gene detection is not considered a gold standard anymore, since the multiplicity of oxacillin-resistance factors requires careful investigation including detection of different resistance genetic markers for correct interpretation of heterogeneous phenotypes expressed by *Staphylococcus* spp. strains. The present study evaluated staphylococci species distribution in 100 strains from cats and dogs obtained from veterinary clinics in the state of Rio de Janeiro, Brazil. Also, their antimicrobial resistance pattern was established based on phenotypic characteristics and *mec*A and *bla* gene detection.

MATERIALS AND METHODS

Sampling

Clinical specimens from 185 dogs and 34 cats were harvested from distinct infectious sites, during routine care in a small animal veterinary clinic of Federal Rural University of Rio de Janeiro (HVPA-UFRRJ) and veterinary care units from different regions of Rio de Janeiro state, Brazil, between 2006 and 2010. The samples were obtained from canine external otitis, skin lesions, urinary and respiratory tract infections, pyometra, periodontitis and conjunctivitis. Bacterial identification and antimicrobial susceptibility assays were performed at the Veterinary Bacteriology Laboratory of Federal Rural University of Rio de Janeiro (LABAC-VET/UFRRJ). Results were sent back to the attending veterinarians to help in diagnosis and therapeutic procedures.

Staphylococcus spp. identification

Samples were inoculated primarily in blood agar (blood agar base enriched with 5% sheep blood) and incubated at 35°C for 24 h. Then the isolates were submitted to the routine microbiological diagnostics, including inoculation in selective medium for analysis of cultural properties, catalase and coagulase production, hemolysis pattern, maltose and D-mannitol fermentation, acetoin production and nitrate reduction (Winn et al., 2006). After phenoltypic identification, isolates were submitted to polymerase chain reaction for 16S rRNA to confirm the presence of *Staphylococcus* spp. (Zhang et al., 2004). Furthermore, PCR amplification of endonuclease genes (*nuc1* and *nuc2*) was performed to identify *S. hyicus.* Strains of *S. pseudintermedius* and *S. aureus* were characterized by the amplification of *nuc3* and *nuc4* genes and 23S rDNA, respectively (Sazaki et al., 2010; Silva et al., 2003). The following standard strains were used as controls: ATCC 29213 *S. aureus*, ATCC 29663 *S. intermedius*, *S. hyicus* 5368 and *S. schleiferi* 3975.

Disk diffusion test

Assays were performed using the method and interpretation criteria according to CLSI standards (CLSI, 2011), after overnight incubation at 35°C followed by measurement of inhibition zone diameters. *Staphylococcus* spp. antimicrobial susceptibility was

Table 1. Distribution of Staphylococcus species per sites of infection.

Staphylococcus species	Sites of infection* (number of isolates)										
	CO	SK	UTI	RTI	PY	OMI	CMI	GI	OM	Total	Isolates
Total species											100
S. intermedius	13	11	5	-	1	1	2	-	2		35
S. aureus spp. *aureus*	11	6	1	-	2	1	3	-	-		24
S. hyicus	11	1	1	-	-	-	-	-	-		13
S. aureus spp*. anaerobius*	-	-	-	-	1	-	-	-	-		1
S. schleiferi spp. *coagulans*	2	4	-	-	-	-	-	-	-		6
CPS**	1	4	3	-	1	-	-	-	-		9
S. xylosus	4	2	-	-		1	-	-	-		7
S. hominis	-	2	-	-	1	-	-	-	-		3
S. epidermidis	-	-	-	2	-	-	-	-	-		2

*CO, Canine otitis; SI, skin infection; UTI, urinary tract infection; RTI, respiratory tract infection; PY, pyometra; OMI, oral mucosal infection; CMI, conjunctive mucosal infection; GI, gastrointestinal infection; OM, osteomyelitis. **CPS, coagulase-positive *Staphylococcus* spp. not genetic defined.

evaluated according to the antimicrobial class clinical recommendation for each infectious site, including β-lactamic, macrolide, lincosamide, streptogramin, quinolone, tetracycline and aminoglicosyde. *S. aureus* ATCC25923 and *Escherichia coli* ATCC25922 were used as quality controls.

Oxacillin susceptibility tests

Resistance to oxacillin was determined according to phenotypic tests recommended by the CLSI (2013). The disk diffusion test was applied using oxacillin (1 µg) and cefoxitin (30 µg) disks (Sensifar-Cefar®), in an agar screen plate containing 6 µg /ml of oxacillin with Müller Hinton agar supplemented with NaCl (4% w/v; 0.68 mol/L). *S.aureus* ATCC29213 was used as quality control.

β-lactamase production

The nitrocefin disk test was applied to detect *Staphylococcus* spp. strains that produce chromogenic β-lactamase, in accordance with the CLSI standard (CLSI, 2013). *S.aureus* ATCC29213 was used as quality control.

DNA extraction and PCR analysis

A 1.5-ml overnight culture of a single *Staphylococcus* colony was centrifuged for 30 s at 14,000 rpm, washed twice in 1 mL TE buffer (10 m*M* Tris HCl, pH 8.0; 1 m*M* EDTA; 100 m*M* NaCl). The resulting pellet was resuspended in 400 µL of TE buffer including 5 µL of lysostaphin (stock concentration 1 µg/mL; Sigma-Aldrich) and incubated for 30 min at 37°C. Lysis was completed by 10 min of water incubation at 100°C. PCR assays of *mec* and *bla* gene complexes were performed using the primers and respective program previously described (Petinaki et al., 2001). The reaction was performed in a final volume of 20 µL of mixture containing PCR buffer (10 mM TrisHCl, pH 9.0; 50 mM KCl, and 0.1% Triton X-100), 3.5 mM $MgCl_2$, 250 µM of each of the deoxynucleoside triphosphates, 3.0 µM of each gene-specific primer, 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI) and 5 µL of template. Amplicons were detected by 1.5% agarose gel, stained with ethidium bromide solution (0.5 mg/mL) and examined under a UV transilluminator (UvTrans). *S.aureus* ATCC43300 was used as quality control.

RESULTS AND DISCUSSION

A total of 100 *Staphylococcus* spp. isolates were phenotypically characterized, as the following species: *S. aureus* spp. *aureus* (n=24), *S. pseudintermedius* (n=44), *S. aureus* spp. *anaerobius*, (n=1), *Staphylococcus schleiferi* spp. *coagulans* (n=6) and coagulase-negative *Staphylococcus* spp. (CoNS) (n=12), represented by *S. xylosus*, *S. epidermidis* and *S. hominis*. PCR amplification of endonuclease genes (*nuc1* and *nuc2*) identified 13% (13/100) *S. hyicus*. Also 35% *S. pseudintermedius* (35/100) and 24% *S. aureus* (24/100) were genetically characterized by PCR amplification of *nuc3* and *nuc4* genes and 23S rDNA, respectively. Table 1 presents *Staphylococcus* species distribution considering the different sites of infection.

These results suggest widespread distribution of staphylococci species in pet animals' infectious sites and corroborate the importance of correct microbiological identification. In veterinary medicine, other CoPS have frequently been misidentified as *S. aureus* strains, due to their common phenotypic traits.

Unfortunately, there has been no reliable phenotypic method to distinguish among CoPS species in veterinary clinical laboratories. In the present study, we used PCR of the thermonuclease (*nuc*) genes to improve the identification of staphylococcal species, as recommended by Sasaki et al. (2010).

Empirical treatment based on *Staphylococcus aureus* as the traditional staphylococci pathogen leads to therapeutic failures and antimicrobial resistance development. As a matter of fact, the latest report from CLSI (2013) established different criteria for oxacillin-

resistance evaluation for *S. aureus* and S. *pseudintermedius* due to their importance in clinical therapy.

In the present study, the disk diffusion test was performed to form a resistance panel for the most used antimicrobial class in staphylococci infections. We detected a low level of resistance to the associations between ampicillin and sulbactam (2%) and amoxicillin and clavulanic acid (5%). This was also true for the antibiotics with restricted use due to high cost, such as imipenen (2%) and linezolide (7%) (Table 2). Tenover et al. (2007) comparing the most commonly used susceptibility testing methods challenged with linezolid-non susceptible staphylococci, concluded that generally the problem was much greater in the non-detection of resistance rather than a possible overcalling of resistance. For this study, the criterium adopted to report linezolide resistance followed CLSI standards that consider any discernible growth within the zone of inhibition as indicative of resistance to linezolide. The highest level of resistance was recorded for β-lactamic antimicrobials, such as penicillin (78%), ampicillin (66%) and ceftriaxone (64%). For the other compounds tested, the resistance was intermediate.

Despite the well-known reduction in β-lactamic efficacy, this class of antimicrobials remains widely used, mainly because of the low treatment cost. Most β-lactamic bacterial resistance mechanisms, such as β-lactamases production and transmembrane permeability reduction can interfere in the activity of other antimicrobial classes when staphylococci species are involved in the infection's etiology, justifying the importance of adopting a susceptibility test before prescribing the drug treatment regimen. Table 2 presents an antimicrobial resistance panel for the antimicrobial classes used to treat staphylococcus infections.

All *Staphylococcus* spp. isolates were submitted to oxacillin and cefoxitin disk diffusion tests. Resistances of 37% (37/100) and 53% (53/10) were detected, respectively. The oxacillin agar screen test detected 57% (57/100) resistance. The evaluation of phenotypic tests linked to *mec*A gene complex detection yielded 15 different oxacillin-susceptibility profiles (Table 3), confirming *Staphylococcus* spp. as a heterogeneous resistance phenotype. Because of this disparity and the difficulty to establish reliable parameters, the CLSI standard procedures are under constant revision. For the prediction of phenotypic oxacillin resistance, some important new recommendations were recently published (CLSI, 2013). The cefoxitin disk test should be performed for *S. aureus* and CoNS and diffusion zones larger than 21 and 24 mm, respectively, should be reported as oxacillin-resistant. Oxacillin disk diffusion is considered the best way to detect *mec*A mediated resistance in *S. pseudintermedius* and should be performed instead of cefoxitin diffusion, which is not considered predictive for *mec*A mediated resistance in this species anymore (CLSI, 2013).

The detection of the *mecA* gene used to be considered a gold standard for the prediction of oxacillin resistance in *Staphylococcus* spp. Recently, the CLSI (2013) changed this criterion, considering the different mechanisms underlying this resistance. In this study, from all isolates tested by PCR assay, just 25% (25/100) were positive for this gene. Among *Staphylococcus* species, a total of 36% (8/22) were *Staphylococcus aureus mecA* +. Other *mecA*+ isolates corresponded to 23% (8/35) *S. intermedius* and 23% (3/13) *S. hyicus*. Coagulase-negative *Staphylococcus* spp. presented a total of 50% (6/12) *mecA*-positive isolates, and the species were: *S. xylosus* (3/6), *S. epidermidis* (2/6) and *S. hominis* (1/6). Results of the *mec* regulatory system assays are presented in Table 2. It was possible to detect the whole *mec* genic complex, *mecA-mecI-mecR1*, in 16% (4/25) of *mec*A+ staphylococcus spp. In this study, the strains presenting the whole regulatory complex were phenoltypically oxacillin-resistant in all performed assays. In contrast, 16% (4/25) of *Staphylococcus* isolates that tested positive to *mecA-mecI* genes presented an oxacillin-susceptible pattern. This might be related to the strong repressive activity exerted by *mecI* in the *mecA* gene. Seven isolates (28%) tested positive to *mecA-mecR1* genes and were also phenotypically oxacillin-resistant, probably due to *mecRI* repression in the *mecI* gene. Mckinney et al. (2001) remarked that the *mecR1* gene is correlated to a membrane signal transduction system which recognizes the extracellular presence of a β-lactamic antimicrobial and induces the transcription of the *mecA* gene. Ten (10/25) *Staphylococcus* spp. isolates tested positive only for the *mecA* gene and were phenotypically oxacillin resistant, as expected by the expression of constitutive PBP2A (Li et al., 2007). These divergent results in the detection of the mec complex can be related to gene mutation or deletion, as described by Katayma and Hiramatsu (2001). It is possible that rare but significant differences in primer annealing sites causes impairment of whole *mec* gene complex detection as observed by Melo et al. (2014) in bovine isolates.

The *mecA* gene is also associated with the multidrug resistance phenotype. In this study, we considered as multidrug resistant the strains that presented resistance to at least three different antimicrobial classes, such as beta-lactamics, quinolones, cephalosporins, macrolides, lincosamide and aminoglicosyde. This profile was detected in 68% (17/25) of mecA + isolates. Among these multiresistant isolates, 41% (7/17) were *S. aureus,* 35% (6/17) *S. intermedius* and 24% (4/17) CNS (Table 3). The spread of multiresistant *Staphylococcus* spp. strains among animals has been investigated worldwide in recent decades and has been blamed on selective pressure exerted by indiscriminate antimicrobial use in veterinary medicine. This resistance provides a selective advantage for *Staphylococcus* spp. infection and colonization, limiting the efficacy of the antimicrobials

Table 2. Resistance panel to antimicrobial classes used in staphylococcus infections.

Antimicrobial class	% (n) Resistant isolates
β-lactamics	
Ampicillin (10 μg)	66% (66/100)
Penicillin (10 UI)	78%(78/100)
Oxacillin (1 μg)	37%(37/100)
β-lactamics + β-lactamase inhibitor	
Ampicillin + Sulbactam (10/10 μg)	2%(2/100)
Amoxicillin + Clavulanic (20/10 μg)	5%(5/100)
Cephalosporins	
Cefoxitin (30 μg)	53%(53/100)
Cefalotin (30 μg)	28%(28/100)
Ceftriaxone (30 μg)	64%(64/100)
Carbapenem	
Imipenem (10 μg)	2%(2/100)
Macrolides	
Azithromycin (15 μg)	53%(53/100)
Erythromycin (15 μg)	48%(48/100)
Lincosamide	
Clyndamicin (2 μg)	57%(57/100)
Quinolones	
Ciprofloxacin (5 μg)	18%(18/100)
Enrofloxacin (10 μg)	15%(15/100)
Norfloxacin (10 μg)	12%(12/100)
Aminoglicosyde	
Tobramicin (10 μg)	45%(45/100)
Gentamycin (10 μg)	22%(22/100)
Folate Pathway Inhibitor	
Sulfamethoxazole+trimetoprim (1,25 μg/23,75 μg)	37%(37/100)
Oxazolidinone	
Linezolide (30 μg)	7%(7/100)
Tetracyclines	
Tetracycline (30 μg)	30%(30/100)
Streptogramin	
Quinupristin/dalfopristin (10 μg/10 μg)	57%(57/100)

available for therapeutic procedures (Souza et al., 2012). Table 4 displays the antimicrobial resistance profile of 25 the *mecA*-positive *Staphylococcus* strains.

Interpretative antimicrobial susceptibility tests can provide clues to the mechanism underlying resistance. Beta-lactamic resistance due to beta-lactamases

Table 3. Oxacillin resistance and *mec* genes detection profiles among 100 *Staphylococcus* isolates.

Profile (n isolates)		ODD	AS	CFO	*mecA*	*mecI*	*mecR1*
Staphylococcus mec - isolates							
1(18)		S	S	S	-	-	-
2(14)		S	S	R	-	-	-
3(14)		R	R	S	-	-	-
4(20)		S	R	S	-	-	-
5(6)		R	S	R	-	-	-
Staphylococcus mec + isolates							
6(5)	SCN (n=3), *S.int.* (n=1), *S.hy.*(n=1)	R	R	R	+	-	-
7(5)	*S.int.* (n=4), *S.hy.*(n=1)	S	R	R	+	-	-
8(5)	*S.int.* (n=3), *S.hy.*(n=1), SCN (n=1)	S	R	R	+	-	+
9(4)	*S. au.*(n=4)	S	S	S	+	+	-
10(2)	*S. au.*(n=1),SCN (n=1)	R	R	R	+	-	+
11(2)	*S.int.* (n=2)	S	S	S	-	+	-
12(1)	SCN (n=1)	S	S	S	-	-	+
13(1)	*S. au.*(n=1)	S	R	S	+	+	+
14(1)	*S. au.*(n=1)	S	R	R	+	+	+
15(2)	*S. au.*(n=1), SCN (n=1)	R	R	R	+	+	+

ODD, Oxacillin disk diffusion; AS, Agar screen; CFO, Cefoxitin disk diffusion; SCN, Coagulase-negative *Staphylococcus* spp.; *S. au.*, *Staphylococcus aureus*; *S.int.*, *Staphylococcus intermedius*; *S.hy.*, *Staphylococcus hyicus*.

Table 4. Antimicrobial resistance profile of 25 *mecA*-positive *Staphylococcus* spp.

Profile (n)	*mecA*	OXA	CFO	PENG	ASB	AMP	CIP	ENO	ERI	AZI	CLI	GEN	LNZ
1(6)	+	R	R	R	S	R	S	R	R	R	R	S	S
2(4)	+	R	R	R	S	R	R	R	R	R	R	S	S
3(2)	+	S	S	R	S	S	S	S	R	S	S	S	S
4(2)	+	S	R	R	R	R	S	S	R	R	R	S	S
5(1)	+	R	R	R	S	R	S	S	R	S	S	S	S
6(1)	+	R	R	R	S	R	S	S	S	R	R	S	S
7(1)	+	R	R	R	S	R	R	S	S	R	R	S	S
8(1)	+	S	S	R	S	R	S	S	R	R	R	R	S
9(1)	+	R	R	R	S	R	S	S	S	S	S	S	S
10(1)	+	S	R	R	S	R	S	S	S	S	S	S	S
11(1)	+	R	R	S	S	S	S	S	R	R	R	S	S
12(1)	+	S	S	R	S	R	S	S	S	S	S	S	S
13(1)	+	S	R	R	S	R	R	S	S	S	S	S	S
14(1)	+	S	R	R	S	R	S	R	S	S	S	S	S
15(1)	+	S	R	R	S	S	S	S	S	S	S	S	S

OXA, oxacillin; CFO, cefoxitin; PENG, penicillin G; ASB, ampicillin+sulbactam; AMP, ampicillin; CIP, ciprofloxacin; ENO, enrofloxacin; ERI, eritrhomycin; AZI, azithromicyn; CLI, clyndamicin;; GEN, gentamycin; LNZ, linezolide; n= nunber of isolates.

production is easily noticed when the resistant strain presents a susceptible pattern to oxacillin and to the association of antimicrobial plus beta-lactamase inhibitors, such as clavulanic acid or sulbactam. The CLSI (2011) recommends the nitrocefin-based test, which detected that 38% (38/100) of *Staphylococcus* spp. were

Table 5. Pheno and genotypic profile of 32 *Staphylococcus* spp. positive to *bla* genes.

Profile (n isolates)	Nitrocefin test	*blaZ*	*blaI*	*blaRI*	*mecA*
1(7)	+	+	-	-	-
3(2)	+	+	-	-	+
4(7)	-	+	+	-	+
8(7)	-	+	+	-	-
11(5)	+	+	+	-	-
12(3)	+	+	+	+	-
13(1)	-	-	+	-	-

β-lactamase producers. As expected, these isolates were resistant to β-lactamic antibiotics, such as penicillin, ampicillin and amoxicillin, whereas they were sensitive to the β-lactamic plus β-lactamase inhibitor associations, such as ampicillin+sulbactam and amoxicillin+clavulanate. Also, 32% (32/100) *Staphylococcus* spp. tested positive for *bla* genes (Table 5). β-lactamases is widespread among animals and it seems to be a prevalent mechanism in resistant staphylococci strains (Mckinney et al., 2001).

Among nitrocefinase producing isolates, nine (9/38) *Staphylococcus* spp. were positive only for the *blaZ* operator gene. Seven (7/9) were *blaZ*-positive-*mecA*-negative *Staphylococcus* spp. and phenotypically oxacillin resistant, suggesting that β-lactamase production was responsible for the observed phenotype. Two isolates tested *blaZ-mecA*-+, being able to express both β-lactamase and PBP2a. The whole *bla* gene complex, *blaZ-blaI-blaR1*, was detected in 7.8% (3/38) of the nitrocefinase-positive isolates. These isolates were oxacillin resistant but *mecA* negative, pointing to the involvement of β-lactamase in this resistance. The extracellular presence of β-lactamic antimicrobial triggers a transduction signal system constituted by BlaR1 transmembrane protein, which signals removal of the *blaI* repressive component that is located between *blaRI* and *blaZ* genes, starting *blaZ* transcription and consequently β-lactamase production, meaning a resistant phenotype (Mckinney et al., 2001). The *blaZ-blaI* genes were detected in 36.8% (14/38) of *Staphylococcus* spp. isolates. From these 14 isolates, 28.5% (4/14) tested negative for *mecA* and nitrocefinase, and presented an oxacillin-susceptible profile, confirming the inhibitory activity of the *blaI* gene in β-lactamase production. In contrast, 42.8% (6/14) tested positive for *mecA* and were also oxacillin susceptible. Mckinney et al. (2001) reported homology of the *mecA* gene to the upstream sequence of the *blaZ* gene. So, distinct mechanisms of oxacillin resistance can also be controlled by the *blaI-blaR1* regulatory system and the *blaI* gene regulates the BlaI membrane system as well as β-lactamase and PBP2a synthesis inhibition. Nine *Staphylococcus* spp. isolates that were *blaZ-blaI*-positive tested negative for *mecA* and presented resistance to oxacillin. This suggests the existence of other resistance mechanisms, such as different classes of PBPs (PBP3 and PBP4). Because of this heterogeneity, the most recent CLSI revision (CLSI, 2013) established new parameters and recommended oxacillin and cefoxitin disk diffusion plus oxacillin agar screen tests to evaluate mecA-mediated resistance. Also, tests should be performed to detect beta-lactamase production. It is no longer possible to consider *mec*A as a gold standard test in beta-lactamic resistance detection.

Conclusion

The widespread distribution of staphylococcus species in pet animal infection sites indicates the importance of correct microbiological identification. *Staphylococcus* strains have potential ability to develop different mechanisms of oxacillin resistance, resulting in a heterogeneous phenotype profile. The oxacillin resistance detected in this study is associated with the existence of different regulatory systems, such as *mec* and *bla* genic complexes, which can either be present or absent in the *Staphylococcus* spp. chromosome and can act in amplified (synergetic) or divergent (non-cumulative) ways. These resistance mechanisms were detected among *Staphylococcus* spp. strains isolated from pet animal infection sites, contributing to reduction of antimicrobial therapeutic efficacy and spread of resistance. Nevertheless, it seems that the action of β-lactamases is a prevalent mechanism in the development of resistant staphylococcus strains.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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REFERENCES

Bannoehr J, Guardabassi L (2012). *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Vet Dermatol. 23(4):253-66.

Clinical and Laboratory Standards Institute (CLSI) (2011). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standards. CLSI document M100-S20, Wayne, Pa, USA.

Clinical and Laboratory Standards Institute (CLSI) (2013). Performance Standards for Antimicrobial Disk and Diluition Susceptibility Tests for Bacteria Isolated From Animals; Approved Standards - 4th Edition,

CLSI document VET01-A4. Wayne, PA, USA.
Devriese LA, Vancanneyt M, Baele M. (2005). *Staphylococcus intermedius* sp. nov., a coagulase positive species from animals. Int. J. Syst. Evol. Microbiol. 55: 1569-1573.
Frank LA, Loeffler A. (2012). Meticillin-resistant *Staphylococcus pseudintermedius*: Clinical challenge and treatment options. Vet. Dermatol. 23:283-56.
Futagawa-Saito K, Ba-Thein W, Sakurai N, Fukuyasu T (2006). Prevalence of virulence factors in *Staphylococcus* intermedius isolates from dogs and pigeons. BMC Vet. Res. 2:4.
Hanselmann BA, Kruth S, Weese S (2008). Methicillin-resistant staphylococcal colonization in dogs entering a veterinary teaching hospital. Vet. Microbiol. 126:277-281.
Ito T, Katayama Y, Asda K, Mori N, Tsutsumimoto K, Tiensasitorn C, Hiramatsu K (2001). Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *S. aureus*. Antimicrob. Agents Chemother. 45:1323-1336.
Jansen MD, Box ATA, Fluit AC (2009). *SCCmec* Typing in methicillin-resistant *Staphylococcus aureus* strains of animal origin. Emerg. Infect. Dis. 15(1):136-137.
Katayama Y, Ito T, Hiramatsu K (2001). Genetic organization of the chromosome region surrounding *mecA* in clinical staphylococcal strains: role of *IS431*-mediated *mecI* deletion in expression of resistance in *mecA*-carrrying, low-level methicillin resistant *Staphylococcus haemolyticus*. Antimicrob. Agents Chemother. 45(7):1955-1963.
Li XZ, Mehrotra M, Ghimire S, Adewoye L (2007). β-lactam resistance and β-lactamases in bacteria of animal origin . Vet. Microbiol. 121(3-4):197-214.
McCarthy AJ, Lindsay JA, Loeffler A (2012). Are all meticillin-resistant *Staphylococcus aureus* (MRSA) equal in all hosts? Epidemiological and genetic comparison between animal and human MRSA. Vet Dermatol. 23(4): 267-275, e53-4.
Mckinney TK, Sharma VK, Craig WA, Archer GL (2001).Transcription of the gene mediating methicilin resistance in *Staphylococcus aureus* (*mecA*) is corepressed but not conduced by cognate *mecA* and β-lactamse regulators. J. Bacteriol. 183:6862-6868.
Melo DA, Coelho IS, Motta CA, Rojas ACM, Dubenczuk F, Coelho SMO, Souza MMS (2014). Evaluation of *mec*A gene sequences and detection of homologue *pbp*D correlated to phenotypic oxacilin resistance in *Staphylococcus sciuri* group. Braz. J. Microbiol. Submitted.
Morgan M (2008). Methicillin-resistant *Staphylococcus aureus* and animals: zoonotic or humanosis. J. Antimicrob. Chemother. 62:1181-1187.
Petinaki E, Arvaniti A, Dimitracopoulos G, Spiliopoulou I (2001). Detection of *mecA*, *mecR1* and *mecI* genes among clinical isolates of methicilin-resistant staphylococci by combined polymerase chain reactions. J. Antimicrob. Chemother. 47:297-304.
Rosato AE, Kreiswirth BN, Graig WA, Eisner W, Climo MW, Aecher GL (2003). *mecA-BlaZ* corepressors in clinical *Staphylococcus aureus* isolates. Antimicrob. Agents Chemother. 47:1463-1466.
Rowland SJ, Dyke KJ (1990). Tn*552*, a novel transposable element from *Staphylococcus aureus*. Mol. Biol. 4:965-975.
Sasaki T, Tsubakishita S, Tanaka Y, Sakusabe A, Ohtsuka M, Hirotaki S, Kawakami, Fukata T, Hiramatsu K (2010). Multiplex-PCR method for species identification of coagulase-positive Staphylococci. J. Clin. Microbiol. 48(3): 765-769.
Silva WP, Silva AJ, Macedo MRP, Araújo MR, Mata MM, Gandra EA (2003). Identification of S*taphylococcus aureus*, S. *intermedius* and *S. hyicus* by Pcr amplification of *coa* and *nuc* genes. Braz. J. Microbiol. 34(1):125-127.
Souza MMS, Coelho SMO, Pereira IA, Soares LC, Pribul BR, Coelho IS (2012). Antibiotic Resistance in Staphylococcus Species of Animal Origin. In: Marina Pana. Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium.
Tenover FC, Williams PP, Stocker S, Thompson A, Clark LA, Limbago B., Carey RB, Poppe SM, Shinabarger D, McGowan-Jr JE (2007). Accuracy of Six Antimicrobial Susceptibility Methods for Testing Linezolid against Staphylococci and Enterococci. J. Clin. Microbiol. 45(9): 2917-2922.
Winn W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, Woods G (2006). Koneman's Color Atlas and Diagnostic Microbiology. 6ed, Lippincott Williams & Wilkins.
Zhang K, Sparling J, Chow BL, Elsayed S, Hussain Z, Church DL, Gregson DB, Louie T, Conly JM (2004). New Quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative Staphylococci. J. Clin. Microbiol. 42 (11): 4947-4955.

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Virulence factors and antibiotic resistance patterns of uropathogenic *Escherichia coli*

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Urinary tract infections (UTIs) are one of the most common infections in humans and the commonest cause is uropathogenic *Escherichia coli* (UPEC). UPEC possess various virulence factors which enable them to survive and grow in urine and other extra-intestinal environments. Similarly, avian pathogenic *E. coli* (APEC) are known for their ability to cause extra-intestinal diseases in birds. Since APEC and UPEC may encounter similar challenges when establishing infection in these locations, they may share a similar content of virulence genes and capacity to cause disease. In this study, 40 UPEC isolates were obtained from patients with suspected UTIs. Multiplex polymerase chain reaction (PCR) was then used to screen the 40 UPEC isolates for 12 virulence genes usually associated with APEC isolates. The *iutA* (35%), *fimH* (32.5%), *vat* (17.5%), *sitA* (17.5%), *sitD* (15%), *hlyF* (12.5%), *pstB* (10%) and *frz* (7.5%) genes were detected. None of the isolates had the *kpsM*, *ompT, uvrY* and *sopB* genes. Antibiotic resistance patterns were also determined for all 40 isolates. A high resistance to ampicillin (90%) and tetracycline (75%) accompanied by a high sensitivity to gentamycin (82.5%) and nitrofurantoin (62.5%) was observed. Eleven multi-drug resistance patterns were observed in 65% (26/40) of the isolates. The studied UPEC isolates were shown to possess APEC associated virulence genes at low percentage frequencies suggesting a slight overlap in virulence genotypes. Antibiotic resistance patterns suggest surveillance programs to monitor drug resistance should be put in place.

Key words: Uropathogenic *Escherichia coli*, virulence genes, multiplex polymerase chain reaction (PCR), antibiotic resistance, Zimbabwe.

INTRODUCTION

Pathogenic strains of *Escherichia coli* are responsible for urinary tract infections (UTIs) in humans. Despite the great wealth of knowledge on *E. coli*, it is still the commonest urinary tract pathogen causing 60–90% of infections (Cheesbrough, 2006; Barati et al., 2011; Perera et al., 2012).

In order to colonize and establish a UTI, uropathogenic *E. coli* strains take advantage of an assortment of virulence properties (Slavchev et al., 2009). By definition, virulence genes/factors (VFs) refer to the properties (gene products) that enable a microorganism to establish itself

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on or within a host of a particular species and enhance it's potential to cause disease (Johnson, 1991). VFs include bacterial toxins, cell surface proteins that mediate bacterial attachment, cell surface carbohydrates and proteins that protect a bacterium and hydrolytic enzymes that may contribute to the pathogenicity of the bacterium (Momtaz et al., 2013).

Recently, work on *E. coli* has sought to investigate the relationship between extra-intestinal pathogenic *E. coli* (ExPEC) strains (Ewers et al., 2007; Moulin- Schouleur et al., 2007; Johnson et al., 2008). These include mainly avian pathogenic *E. coli* (APEC) which causes colibacillosis in poultry, UPEC and neonatal meningitis *E. coli* (NMEC). Comparisons of these isolates have generally revealed that some overlaps exist in serogroups, virulence genotypes and abilities to cause disease in certain animal models (Rodriguez- Siek et al., 2005; Ewers et al., 2007; Moulin-Schouleur et al., 2007).

Work on virulence genes in the western world and most of Asia is on the rise, but in Africa very little information seems to be available (van der Westhuizen and Bragg, 2012; Randall et al., 2012). This study was undertaken to screen for avian-related virulence genes in UPEC. Use of the avian-related virulence genes was meant to allow us to compare virulence genes found in *E. coli* responsible for human UTIs to APEC in diseased chicken.

The 12 virulence genes that were used for molecular characterization of uropathogenic *E. coli* in the present study included the *frz* operon (Rouquet et al., 2009); vacuolating autotransporter toxin, *vat* (Parreira and Gyles, 2003); type 1 fimbrial adhesion gene, *fimH* (Mellata et al., 2003); capsule formation transporter gene, *kpsM* (Pavelka et al., 1991); the gene *ompT* (Cavard and Lazdunski, 1990); the *sitA* and *sitD* genes which are part of the sitABCD system (Runyen-Janecky et al., 2003); a putative avian haemolysin gene, *hlyF*; an aerobactin siderophore receptor gene, *iutA* (Williams and Warner, 1980; Morales et al., 2004); a transcriptional regulator of iron uptake gene in APEC, *uvrY* (Li et al., 2004); the gene *pstB* (Lamarche et al., 2005) and the plasmid partitioning protein encoded by *sopB* which is common in various plasmids.

Antibiotic resistance/sensitivity patterns are important in the selection of antibiotics that can be used as combinations in the treatment of urinary tract infections. In a recent report by the World Health Organisation (WHO) it was mentioned how we are headed for a post-antibiotic era, in which common infections and minor injuries which have been treatable for decades will once again kill. The report draws on data from 114 countries, and focuses on antibiotic resistance in bacteria that cause common but serious diseases such as sepsis, diarrhea, pneumonia, urinary tract infections and gonorrhea (WHO, 2014). Because of the lack of an effective vaccine to combat UTIs, antimicrobial therapy remains crucial for the control of urinary tract infections. Another major objective of this study was to provide current antibiotic resistance patterns of UPEC isolates from Zimbabwe.

METHODOLOGY

Sample collection

Between the months of July and August 2013, 86 urine samples were obtained from patients visiting a leading diagnostic Medical Laboratory in Harare. These samples were from both symptomatic and asymptomatic patients being tested for UTIs. Of the 86 urines samples, only 13 were found to have *E. coli* as the cause of bacteriuria. Over a period of three months (October-December 2013) 27 *E. coli* isolates were obtained from a leading diagnostic Medical Laboratory in Bulawayo, these were also from suspected UTIs cases. A total of 40 *E. coli* isolates were used in this study, 13 from Harare and 27 from Bulawayo.

Isolation and identification of *E. coli*

Samples were cultured on cysteine lysine electrolyte deficient agar (CLED agar), blood agar and MacConkey agar (Oxoid, England) and then incubated aerobically at 37°C for 24 h. Biochemical tests were carried out including the Gram stain and the indole, citrate and methyl red test.

Antimicrobial susceptibility testing

The disc diffusion method was used to determine antibiotic susceptibility of the isolates on Mueller Hinton agar (Oxoid, UK). Each isolate was tested for antibiotic susceptibility using a panel of the following antibiotics: nitrofurantoin (50 µg), ampicillin (25 µg), nalidixic acid (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg) and gentamycin (10 µg). All antibiotic disks were from Oxoid, United Kingdom. The plates were incubated at 37°C for 24 h, and inhibition zones were measured. Interpretation of results followed criteria recommended by the Clinical Laboratory Standard Institute (CLSI, 2009).

DNA Isolation

Bacterial strains were subcultured at 37°C overnight in Luria-Bertani (LB) broth (Oxoid, Basingstoke, Hampshire, UK) and genomic DNA was extracted using a standard Phenol-Chloroform method (Sambrook and Russell, 2001). To check for purity, DNA was run along a 1% ethidium bromide stained agarose gel (Sigma-Aldrich, St Louis, USA) with a 1 kb DNA ladder (Thermo Scientific, USA) in TBE buffer for 1 h at 100 V and then viewed using a Uvipro-Silver Gel Documentation System (Uvitec, UK). The concentration of DNA was estimated by comparing the band light intensity to the band intensity on the 1 kb ladder on the Uvipro-Silver Gel Documentation System. DNA concentration of samples ranged from 75 ng/0.5 µg – 100 ng/0.5 µg.

Multiplex PCR for virulence genes

The presence of genes encoding virulence factors was detected using multiplex PCR amplification. Four multiplex PCR assays were used to detect 12 virulence genes (Table 1). The multiplex design was done according to that reported by van der Westhuizen and Bragg (2012) with slight changes in the primer and final magnesium chloride concentrations. The effected changes were done using primer concentrations of 0.5 µM for the *frz*, *sitD*, *fimH*, *ompT*, *iutA*, *pstB* and s*opB* genes and adjusting the final $MgCl_2$ concentration to

Table 1. Final primer concentrations used in the different multiplex PCRs.

Multiplex	Primer set	Conc (µM)	Primer set	Conc (µM)	Primer set	Conc. (µM)	Additional $MgCl_2$ (mM)	Final $MgCl_2$ (mM)
1	*frz*	0.5	*sitD*	0.5	*fimH*	0.5	1	3
2	*sitA*	2.0	*kpsM*	1.0	*vat*	0.5	1	3
3	*ompT*	0.5	*iutA*	0.5	*pstB*	0.5	1	3
4	*sopB*	0.5	*uvrY*	1.0	*hlyF*	0.5	1	3

Table 2. Frequency of 12 virulence genes in 40 uropathogenic *E. coli* isolates.

Name of gene	Primer name	Frequency (%)
Aerobactinsiderophore receptor	*iutA*	35
Type 1 fimbrial adhesin	*fimH*	32.5
Vacuolating autotransporter toxin	*vat*	17.5
SitABCD system	*sitA*	17.5
SitABCD system	*sitD*	15
Putative avian haemolysin	*hlyF*	12.5
PstSCAB system	*pstB*	10
frz operon	*frz*	7.5
APEC virulence regulator	*uvrY*	0
Capsule-protein transport of polysaccharides	*kpsM*	0
Episomal outer membrane protease	*ompT*	0
Plasmid partitioning protein	*sopB*	0

3 mM for all multiplex reactions. The primers used in our study are listed in Table S1 in the supplementary material. All primers used were obtained from Inqaba Biotech, South Africa. Three microliters of each of the DNA samples were mixed with all necessary components for amplification in a 0.2 ml PCR tube (Perkin-Elmer, USA) in a 25 µl reaction. The reaction mixture included 2.5 µl of ×10 PCR Dream Taq buffer (Thermo scientific, USA), 2 µl of dNTPs, 10 mM; 0.25 µl of Dream *Taq* polymerase (Thermoscientific, USA), 5 U/µl, nuclease free water to maintain a total volume of 25 µl. The appropriate primers were added to a maximum primer concentration of 2 µM and the $MgCl_2$ concentration was adjusted to a final concentration of 3 mM as shown in Table 1. Negative controls comprised of a water control. An Applied Biosystems GeneAmp® PCR System 9700 was used for the PCR thermal cycling conditions with an initial denaturation step at 94°C for 5 min, 35 cycles {denaturation 94°C for 30 s, annealing at 63°C for 45 s, extension 72°C for 1 min and 45 s and a final elongation step at 72°C for 10 min. The amplified products were then run along a 1% ethidium bromide stained agarose gel with a 100 bp DNA ladder (Thermo scientific, USA) in TBE buffer for 1 h at 100 V and then viewed using a Uvipro-Silver Gel Documentation System (Uvitec, UK).

The multiplex PCRs described were used to screen for the presence of 12 virulence genes in the UPEC isolates in duplicate. Prevalence of each virulence gene was then calculated (Table 2).

RESULTS

All 40 isolates obtained from the two leading diagnostic laboratories in Harare and Bulawayo were positively identified and confirmed to be *E. coli* through culturing and biochemical tests. After successful DNA isolation and quantification, the DNA of each of the 40 UPEC isolates was subjected to 4 different multiplex PCRs. Each multiplex reaction amplified three virulence gene regions. This was done in order to screen the UPEC isolates for 12 APEC associated virulence genes. The percentage frequency of each gene was then determined and the results are shown in Table 2. The *iutA* gene had the highest presence rate of 35%; followed by *fimH* (32.5%), *vat* (17.5%) *sitA* (17.5%) and *sitD* (15%). The data obtained from electrophoresis agarose gels was used to assign virulence gene profiles to each UPEC isolate, this is summarized in Table 3.

Antibiotic susceptibility testing was done for all 40 UPEC isolates. The isolates were assayed against a panel of six antibiotics. The results shown in Table 4 suggest a high resistance of UPEC to ampicillin (90%) and tetracycline (75%) whilst a high sensitivity to gentamycin (82.5%) and nitrofurantoin (62.5%) was observed. The prevalence of antibiotic resistance phenotypes of all *E. coli* isolates is presented in Table 5. Sixty five percent of the isolates showed resistance to at least 3 antibiotics, and 11 different antibiotic resistance patterns were observed. The most common resistance pattern, exhibited by 10 isolates, was resistance to ampicillin, nalidixic acid, ciprofloxacin and tetracycline (pattern D). This study was sanctioned by the NUST ethical committee and no names were recorded during the study.

Table 3. Presence or absence of expected amplicons and virulence profiles of UPEC isolates

Isolate	Multiplex 1			Multiplex 2			Multiplex 3			Multiplex 4			Total/12	Virulence profile
	frz	*sitD*	*fimH*	*sitA*	*kpsM*	*vat*	*ompT*	*iutA*	*pstB*	*sop B*	*uvrY*	*hlyF*		
1	+	+	+	+	-	+	-	+	-	-	-	-	6	C
2	-	+	+	-	-	-	-	-	-	-	-	-	2	A
3	-	+	+	-	-	-	-	-	-	-	-	-	2	A
4	+	+	+	-	-	+	-	+	-	-	-	-	5	B
5	-	-	-	-	-	-	-	-	-	-	-	-	0	A
6	+	+	+	-	-	-	-	+	-	-	-	-	4	B
7	-	-	-	-	-	-	-	-	-	-	-	-	0	A
8	-	-	-	-	-	-	-	-	-	-	-	-	0	A
9	-	-	-	-	-	-	-	-	-	-	-	-	0	A
10	-	+	+	-	-	-	-	-	-	-	-	-	2	A
11	-	-	-	-	-	-	-	+	-	-	-	-	1	A
12	-	-	-	-	-	-	-	-	-	-	-	-	0	A
13	-	-	-	-	-	-	-	+	-	-	-	-	1	A
14	-	-	-	-	-	-	-	-	-	-	-	-	0	A
15	-	-	-	-	-	-	-	-	-	-	-	-	0	A
16	-	-	-	-	-	-	-	-	-	-	-	-	0	A
17	-	-	+	-	-	-	-	-	-	-	-	-	1	A
18	-	-	+	-	-	-	-	+	-	-	-	-	2	A
19	-	-	-	-	-	-	-	-	-	-	-	-	0	A
20	-	-	-	-	-	-	-	-	-	-	-	-	0	A
21	-	-	-	-	-	-	-	-	-	-	-	-	0	A
22	-	-	-	-	-	-	-	-	-	-	-	-	0	A
23	-	-	-	-	-	+	-	+	-	-	-	-	2	A
24	-	-	-	-	-	-	-	-	-	-	-	+	1	A
25	-	-	-	-	-	-	-	-	-	-	-	-	0	A
26	-	-	-	-	-	-	-	+	-	-	-	+	2	A
27	-	-	-	-	-	-	-	-	-	-	-	-	0	B
28	-	-	-	-	-	-	-	-	-	-	-	-	0	A
29	-	-	-	-	-	-	-	-	-	-	-	-	0	A
30	-	-	+	+	-	+	-	-	+	-	-	+	5	B
31	-	-	+	-	-	-	-	+	-	-	-	-	2	A
32	-	-	+	+	-	-	-	+	-	-	-	-	3	B
33	-	-	-	+	-	-	-	-	-	-	-	-	1	A
34	-	-	-	-	-	-	-	+	-	-	-	-	1	A
35	-	-	+	+	-	+	-	-	+	-	-	-	4	B
36	-	-	-	+	-	-	-	+	+	-	-	-	3	B
37	-	-	-	-	-	+	-	-	+	-	-	+	3	B
38	-	-	-	+	-	+	-	+	-	-	-	+	4	B
39	-	-	-	-	-	-	-	+	-	-	-	-	1	A
40	-	-	+	-	-	-	-	-	-	-	-	-	1	A

Presence (+) or absence (-) of the expected amplicons during the four different multiplex PCRs. Last column indicates virulence profiles assigned to each isolate. A indicates presence of between 0 and 2 virulence genes, B between 3 and 5 genes and C indicate 6 or more virulence genes.

DISCUSSION

Virulence gene profiles

In this study 40 *E. coli* isolates were obtained from patients with suspected UTIs and screened for 12 virulence genes, some of which have been well characterized in previous studies (Karimian et al., 2012; Guiral et al., 2012; Farshad et al., 2012). The *uvrY* gene has not been used to screen for UPEC in previous studies using multiplex PCR tech niques.

Table 4. Antibiotic susceptibility profiles of 40 uropathogenic *E. coli* isolates.

Antibiotic	Concentration (µg)	Resistant no. (%)	Intermediate no. (%)	Susceptible no. (%)
Gentamycin	10	4 (10)	3 (7.5)	33 (82.5)
Nitrofurantoin	50	13 (32.5)	2 (5)	25 (62.5)
Ciprofloxacin	5	19 (47.5)	2 (5)	19 (47.5)
Nalidixic Acid	30	23 (57.5)	1 (2.5)	16 (40)
Tetracycline	30	30 (75)	2 (5)	8 (20)
Ampicillin	25	36 (90)	1 (2.5)	3 (7.5)

Table 5. Antimicrobial resistance patterns of the 40 uropathogenic *E. coli* isolates.

Pattern	No. of isolates	Resistance pattern*
A	2	Amp, Nit, Nal, Cip, Tet, Gen
B	2	Amp, Nit, Nal, Cip, Tet
C	1	Amp, Nal, Cip, Tet, Gen
D	10	Amp, Nal, Cip, Tet
E	1	Amp, Nal, Cip, Gen
F	2	Amp, Nit, Nal, Tet
G	3	Amp, Nit, Tet
H	2	Amp, Nal, Tet
I	1	Amp, Nal, Cip
J	1	Amp, Nit, Nal
K	1	Amp, Cip, Tet

*Amp- ampicillin, Nit- nitrofurantoin, Nal- nalidixic acid, Tet- tetracycline, Cip- ciprofloxacin, Gen- gentamycin.

Similarly, the *frz* and *pstB* genes have been shown to contribute to virulence but are new in the diagnostic context (Li et al., 2004; Lamarche et al., 2005; Rouquet et al., 2009). The multiplex PCRs of this study have an advantage over previous studies as they include these recently discovered virulence genes.

An aerobactinsiderophore receptor gene, *iutA*, which is known to contribute to iron uptake (Williams and Warner, 1980; Morales et al., 2004) was present in 35% of UPEC isolates (Table 2). Presence of the gene in UPEC agrees with other studies (Johnson, 1991; Guiral et al., 2012) that have shown that the bacterial sidephore “aerobactin” is associated with *E. coli* strains which cause pyelonephritis and cystitis. It is an iron sequestration and transport system which enables *E. coli* to grow in iron poor environments such as dilute urine (Johnson et al., 2008).

The type 1 fimbrial adhesin gene, *fimH*, contributes to protection from host heterophils (Mellata et al., 2003). The gene had the second highest frequency (32.5%) and may be useful by UPEC for adhesion to uroepithelial cells. *FimH* is the adhesin protein known to be responsible for binding to mannosylated glycoproteins and is located at the distal tip of the heteropolymeric type I pilus rod (Johnson, 1991; Slavchev et al., 2009). Detection of the gene in the present study agrees with most studies (Tiba et al., 2008; Johnson, 1991; Karimian et al., 2012), however values as high as 79.67% have been reported in other studies (Karimian et al., 2012).

The *sitA* and *sitD* genes which are part of the SitABCD system, and are classified as a bacterial iron transporter (Runyen-Janecky et al., 2003) were also found in 17.5 and 15% of the UPEC isolates respectively. Apart from mediating iron and manganese transport, the SitABCD operon has also been suggested to confer resistance to oxidative stress possibly required during interaction with phagocytes (Sabri et al., 2008). The presence of these genes agrees with other studies (Schouler et al., 2004; Snyder et al., 2004; Rodriguez et al., 2005) who believe that in *E. coli*, SitABCD-encoding genes are associated with clinical strains isolated from extra-intestinal infections from poultry and human UTIs.

The vacuolating autotransporter toxin, *vat* gene which has been shown to induce cytotoxic effects in host cells (Parreira and Gyles, 2003) was present in 17.5% of our isolates. This disagrees with a study by Johnson et al. (2008) who found a prevalence of 62.3% for the *vat* gene in UPEC isolates. A putative avian haemolysin gene, *hlyF*, responsible for iron uptake (Williams and Warner, 1980; Morales et al., 2004) was also amplified in 12.5% of the isolates. A 50.4% presence for *hly A* was observed by Karimian et al. (2012), but our findings agree with recent studies by Farshad et al. (2012) who obtained a 15.62% for hemolysin (*hly*) in UPEC from children in Iran. In another study on 531 UPEC isolates, Johnson et al. (2008) found a prevalence of 5.6% for the *hlyF* gene. Also, according to Johnson (1991) the percentage frequency of genes from the *hly* operon varies with the patient’s condition. *HlyF* and *vat* have been well documented in chickens suffering from colibacillosis (van der Westhuizen and Bragg, 2012) and very little seems to have been published on the genes in UPEC.

The *pstB* gene which is part of the pstSCAB operon, has been shown to increase resistance to polymyxin, rabbit serum and acid shock (Lamarche et al., 2005). We detected it in 10% of the studied UPEC isolates. Some studies (Surin et al., 1985; Rao and Torriani, 1990) suggest that the gene may be responsible for mediating the uptake of phosphate from the outside to the inside of the cell. The *frz* operon was present in 7.5% of the UPEC

isolates and work by Rouquet et al. (2009) suggests that the gene products from the *frz* operon are used by *E. coli* to promote growth in serum during oxygen-restricted conditions. Urine, like serum is also oxygen-restricted, therefore UPEC may use this gene for their survival. Very little has been published on the *pstB* and *frz* operon in UPEC.

Four (*uvrY, sopB, kpsM* and *ompT*) of the twelve genes were not detected in all the 40 *E. coli* isolates (Table 2). Transcriptional regulator of iron uptake genes, *uvrY* (Li et al., 2008) together with the plasmid partitioning protein encoded by *sopB* known to be common in various plasmids were both absent. If a virulence gene in APEC does not have a homologue in UPEC the gene region cannot be amplified except in cases of a zoonotic infection. APEC virulence regulator (*uvrY*) is a VF particularly found in *E. coli* associated with chicken suffering from colibacillosis. Therefore, the gene's absence in all UPEC isolates studied supports work done by Randall et al. (2012), who suggested that *E. coli* strains in diseased chickens are generally different from those causing disease in humans.

Another gene that was absent was the capsule formation transporter gene *kps M* (Pavelka et al., 1991). This finding differs from some previous studies by Momtaz et al. (2013) and Tiba et al. (2008) who indicated presence of the *kpsMT* gene in UPEC. The gene *ompT* which encodes for the episomal outer membrane protease that cleaves colicins (Cavard and Lazdunski, 1990) was also absent in the samples. These findings agree with other recent studies by Karimian et al. (2012) and Momtaz et al. (2013).

Virulence profiles were generated for each *E. coli* isolate used in our study (Table 3).The UPEC isolates were profiled as being 75% profile A, 22.5% profile B and 2.5% profile C. None of the isolates had more than 6 virulence genes. Generally, the UPEC isolates did not possess most of the APEC virulence genes assayed for (Table 3). This suggests that UPEC isolates may have different virulence genotypes than those reported for APEC (Johnson et al., 2008). A potential bias and limitation in our study could have been the use of *E. coli* isolates from both symptomatic and asymptomatic patients and failure to test each isolate using *in vivo* animal models to determine actual virulence.

We also report that *E. coli* isolates which were resistant to all of the six antibiotics had none or only one of the VFs studied. This may suggest that some of the isolates were related, but it however also supports recent studies in Turkey by Giray et al. (2012) who found that *E. coli* strains with low numbers of virulence genes exhibit a high antibiotic resistance. A larger sample size as well as more VFs should be studied to fully investigate this relationship.

Antibiotic susceptibility profiles

The studied UPEC isolates showed a high resistance to ampicillin (90%) and tetracycline (75%) whilst showing a high sensitivity to gentamycin (82.5%) and nitrofurantoin (62.5%) (Table 4). The results agree with previous studies done in the country by Mbanga et al. (2010) who found that UPEC showed the highest resistance to ampi-cillin (84%) whilst showing a low resistance to nitro-furantoin (16%). Our findings are also similar to previous studies from Mexico City (Molina-Lopez et al., 2011), Sri Lanka (Perera et al., 2012), Nigeria (Okonko et al., 2009) India (Mandal et al., 2012) and Iran (Barati et al., 2011) which found a low resistance to nitrofurantoin and a high resistance to ampicillin.

UPEC strains tend to be resistant to drugs that are frequently used. However, gentamycin is inappropriate for frequent use, rather it is commonly used for severe UTIs. Our findings show that most isolates were susceptible to gentamycin (82.5%) but this differs from a study done in Iraq by Chateen et al. (2007), who found UPEC to be highly resistant to gentamycin.

E. coli isolates clearly demonstrated high resistance to most examined antibiotics (Table 5) and this has been reported in other studies (Dromigny et al., 2005). Five percent of UPEC isolates were resistant to all six antibiotics and none of the isolates were susceptible to all the antibiotics. All *E. coli* showed resistance to at least one or more antibiotics. Sixty five percent of the *E. coli* isolates were responsible for the 11 different multi-drug resistance (MDR) patterns (UPEC showing resistance to ≥3 antibiotics) as shown in Table 5. This agrees with recent findings in Iran by Farshad et al. (2012), but differs from other studies were lower levels of MDR isolates have been reported (Linder et al., 2005; Rijavec et al., 2006). The most common pattern was pattern D followed by pattern G (Table 5).

Conclusion

Low percentage frequencies of the studied VFs were detected in UPEC causing UTIs. Half of all UPEC isolates in the present study possessed none, or only one, of the VFs characterized and, as such, it is reasonable to assume that UPEC isolates have different virulence factors than those reported for APEC. However, to fully investigate zoonosis between chicken and humans, virulence genes exclusive to chicken isolates should be used. The antibiotic resistance results show that antibiotic resistance is on the rise and nitrofurantoin should be the drug of choice in Zimbabwe.

Conflict of interest

The author(s) have not declared any conflict of interests.

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REFERENCES

Barati L, Ghezelsofla F, Azarhoush R, Heidari F, Noora M (2011). Antibiotic sensitivity of isolated *E. coli* from pregnant women's urine. J. Gorgan. Univ. Med. Sci. 13(3):101-107.

Cavard D, Lazdunski C (1990). Colicin cleavage by *ompT* protease during both entry into and release from *Escherichia coli* cells. J. Bacteriol. 172:648-652.

Chateen EA, Sheelan AA, Sinan BI, Sajida K (2007). Study of urinary tract infection among pregnant women in Kirkuk. Tikrit Med. J. 13(2):39-43.

Cheesbrough M (2006).Microbiological tests. In: District Laboratory Practice in Tropical Countries Part 2, 2nd edn, New York: Cambridge University Press. pp. 105:133-136

Clinical Laboratories Standards Institute (CLSI). (2009). Performance of standards for antimicrobial disk susceptibility tests; approved standards 10th edn, 29:M02-A10. Wayne, PA.

Dromigny AJ, Nabeth P, Juergens-Behr A, Perrier-Gros-Claude DJ (2005). Risk factors for antibiotic-resistant *Escherichia coli* isolated from community-acquired urinary tract infections in Dakar, Senegal. J. Antimicrob. Chemother. 56:236-239

Ewers C, Li G, Wilking H, Kieβling S, Alt K, Antáo EM, Laturnus C, Diehl I, Glodde S, Homeier T, Bo¨hnke U, Steinru¨ck H, Philipp HC, Wieler LH (2007). Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: How closely related are they? Int. J. Med. Microbiol. 297:163-176.

Farshad S, Ranjbar R, Japoni A, Hosseini M, Anvarinejad M. Mohammadzadegan R (2012). Microbial susceptibility, virulence factors, and plasmid profiles of uropathogenic *Escherichia coli* in children in Jahrom, Iran. Arch. Iran Med. 15(5):312-316.

Giray B, Ucar BF, Aydemir SS (2012). Characterization of uropathogenic *Escherichia coli* strains obtained from urology outpatient clinic of Ege Medical Faculty in İzmir. Turk. J. Med. Sci. 42:1328-1337.

Guiral E, Bosch J, Vila J, Soto SM (2011). Prevalence of *Escherichia coli* among samples collected from the genital tract in pregnant and nonpregnant women: relationship with virulence. FEMS Microbiol. Lett. 314:170-173

Johnson JR (1991). Virulence factors in *Escherichia coli* Urinary Tract Infection. Clin. Microbial. Rev. 4(1):80-128

Johnson TJ, Wannemuehler Y, Doetkott C, Johnson SJ, Rosenberger SC, Nolan LK (2008). Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46:3987-3996.

Karimian A, Momtaz H, Madani M (2012). Detection of uropathogenic *Escherichia coli* virulence factors in patients with urinary tract infections in Iran. Afr. J. Microbiol. Res. 6(39):6811-6816

Lamarche MG, Dozois CM, Daigle F, Caza M, Curtiss R III, Dubreuil JD, Harel J 2005. Inactivation of pst system reduces the virulence of an avian pathogenic *Escherichia coli* O78 strain. Infect. Immun. 73:4138-4145.

Li G, Ewers C, Laturnus C, Diehl I, Alt K, Dai J, Antáo EM, Schnetz K, Wieler LH (2008). Characterization of *ayjjQ* mutant of avian pathogenic *Escherichia coli* (APEC). Microbiology 154: 1082-1093.

Li X, Lockatell, CV, Johnson DE, Lane MC, Warren JW, Mobley HL (2004). Development of an intranasal vaccine to prevent urinary tract infection by *Proteus mirabilis*. Infect. Immun.72:66-75

Linder JA, Huang ES, Steinman, MA, Gonzales R, Stafford RS (2005). Fluoroquinolone prescribing in the United States. Am. J. Med. 118: 259-268.

Mandal J, Acharya NS, Buddhapriya D, Parija CS (2012). Antibiotic resistance pattern among common bacterial uropathogens with a special reference to ciprofloxacin resistant *Escherichia coli.* Indian J. Med. Res. 136: 842-849.

Mbanga J, Dube S, Manyanduki H (2010). Prevalence and drug resistance in bacteria of the urinary tract infections in Bulawayo Province, Zimbabwe. East Afr. J. Public Health 7(3):229-32.

Mellata M, Dho-Moulin M, Dozois CM, Curtiss III R, Brown PK, Arné P, Brée A, Desautels C Fairbrother JM (2003). Role of virulence factors in resistance of avian pathogenic *Escherichia coli* to serum and in pathogenicity. Infect. Immun. 71:536-540.

Molina-López J, Aparicio-Ozores G, Ribas-Aparicio MR, Gavilanes-Parra S, Chávez-Berrocal EM, Hernández-Castro R, Manjarrez-Hernández AH (2011). Drug resistance, serotypes, and phylogenetic groups among uropathogenic *Escherichia coli* including O25-ST131 in Mexico City. J. Infect. Dev. Ctries. 5(12):840-849.

Momtaz H, Karimian A, Madani M, Dehkordi SF, Ranjbar R, Sarshar M, Souod N (2013). Uropathogenic *Escherichia coli* in Iran: Serogroup distributions, virulence factors and antimicrobial resistance properties. Ann. Clin. Microbiol. Antimicrob. 12: 10.1186/1476-0711-12-8.

Morales C, Lee MD, Hofacre C, Maurer JJ (2004). Detection of a novel virulence gene and a *Salmonella* virulence homologue among *Escherichia coli* isolated from broiler chickens. Foodborne Pathog. Dis. 1:160-165.

Moulin-Schouleur MM, Reperant S, Laurent A, Bree S, Mignon-Grasteau P, Germon, Rasschaert D, Schouler C (2007). Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. J. Clin. Microbiol. 45:3366-3376.

Okonko OI, Donbraye-Emmanuel BO, Ijandipe AL, Ogun AA, Adedeji OA, Udeze OA (2009). Antibiotics sensitivity and resistance patterns of uropathogens to nitrofurantoin and nalidixic acid in pregnant women with urinary tract infections in Ibadan, Nigeria. MEJSR 4(2): 105-109.

Parreira RV Gyles LC (2003). A novel pathogenicity island integrated adjacent to the *thrW*tRNA gene of avian pathogenic *Escherichia coli* encodes a vacuolating autotransporter toxin. Infect. Immun. 71(9): 5087-5096

Pavelka MS Jr, Wright LF, Silver RP (1991). Identification of two genes, *kpsM* and *kpsT*, in region 3 of the polysialic acid gene cluster of *Escherichia coli* K1. J. Bacteriol. 173:4603-4610.

Perera J, Randeniya C, Perera P, Gamhewage N, Jayalathaarachchi, R (2012). Research article: Asymptomatic bacteriuria in pregnancy: prevalence, risk factors and causative organisms. Sri Lankan J. Infect. Dis. 1(2):42-46.

Randall L, Wua G, Phillips N, Coldham N, Mevius D, Teale C (2012). Virulence genes in *blaCTX-M Escherichia coli* isolates from chickens and humans. Res. Vet. Sci. 93:23-27

Rao NN, Torriani A (1990). Molecular aspects of phosphate transport in *Escherichia coli*. Mol. Microbiol. 4:1083-1090.

Rijavec M, Starcic-Ergivec M, Ambrozic Augustin J, Reissbrodt R, Fruth A, Krizan-Hergouth V (2006). High prevalence of multidrug resistance and random distribution of mobile genetic elements among uropathogenic*Escherichia coli* (UPEC) of the four major phylogenetic groups. Curr. Microbiol. 53:158-162.

Rodriguez-Siek KE, Giddings CW, Doetkott C, Johnson TJ, Fakhr MK, Nolan LK (2005). Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology 151:2097-2110.

Rouquet G, Porcheron G, Barra C, Re´pe´rant M, Chanteloup KN, Schouler C, Gilot P (2009). A metabolic operon in extraintestinal pathogenic *Escherichia coli* promotes fitness under stressful conditions and invasion of eukaryotic cells. J. Bacteriol. 191(13): 4427-4440.

Runyen-Janecky LJ, Reeves SA, Gonzales EG, Payne SM (2003). Contribution of the *Shigellaflexneri Sit, Iuc,* and *Feo* iron acquisition systems to iron acquisition *in vitro* and in cultured cells. Infect. Immun. 71:1919-1928.

Sabri M, Caza M, Proulx J, Lymberopoulos MH, Bre´e A, Moulin-Schouleur M, Curtiss R, Dozois CM (2008). Contribution of the SitABCD, MntH, and FeoB metal transporters to the virulence of avian pathogenic *Escherichia coli* O78 strain x7122. Infect. Immun. 76:601-611.

Sambrook J, Rusell DW (2001). Molecular Cloning: A Laboratory Manual, 3rd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Schouler C, Koffmann F, Amory C, Leroy-Setrin S, Moulin-Schouleur M (2004). Genomic subtraction for the identification of putative new virulence factors of an avian pathogenic *Escherichia coli* strain of O2 serogroup. Microbiology 150: 2973-2984.

Slavchev G, Pisareva E, Markova N (2009). Virulence of uropathogenic *Escherichia coli.* J. Cult. Collect. 6:3-9.

Snyder JA, Haugen BJ, Buckles EL, Lockatell CV, Johnson DE, Donnenberg MS, Welch RA, Mobley HL (2004). Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. Infect. Immun. 72:6373-6381.

Surin BP, Rosenberg H, Cox BG (1985). Phosphate-specific transport system of *Escherichia coli*: nucleotide sequence and gene-polypeptide relationships. J. Bacteriol. 161:189-198.

Tiba RM, Yano T, Leite SD (2008). Genotypic characterization of virulence factors in *Escherichia coli* strains from patients with cystitis. Rev. Inst. Med. Trop. S Paulo 50(5):255-260.

van der Westhuizen AW, Bragg RR (2012). Multiplex polymerase chain reaction for screening avian pathogenic *Escherichia coli* for virulence genes. Avian Pathol. 41(1):33-40.

Williams PH, Warner PJ (1980). ColV plasmid-mediated, colicin Vindependent iron uptake system of invasive strains of *Escherichia coli*. Infect. Immun. 29:411-416.

World Health Organisation (WHO). (2014). Antimicrobial resistance: global report on surveillance. http://www.who.int/drugresistance/documents/surveillancereport/en/ (accessed 23/07/14).

Table S1. Primers used for amplifying regions in APEC virulence genes.

Primer name	Name of targeted region/gene	Primer Sequence (5' – 3')	Source of Primer Sequence	Expected amplicon size (bp)
sitA-F *sitA*-R	SitABCD system	Forward primer: CGCAGGGGGCACAACTGAT Reverse primer: CCCTGTACCAGCGTACTGG	Sabri et al. (2008)	661
sitD-F *sitD*-R	SitABCD system	Forward primer: CTGTGCGCTGCTGTCGGTC Reverse primer: GCGTTGTGTCAGGAGTAC	Sabri et al. (2008)	571
ompT-FP *ompT*-RP	Episomal outer membrane protease	Forward primer: TCATCCCGGAAGCCTCCCTCACTACTAT Reverse primer: TAGCGTTTGCTGCACTGGCTTCTGATAC	Johnson et al. (2008)	496
fimH-FP *fimH*-RP	Type 1 fimbrial adhesin fimH	Forward primer: GGATAAGCCGTGGCCGGTGG Reverse primer: CTGCGGTTGTGCCGGAGAGG	Van der Westhuizen and Bragg (2012)	331
frz-FP *frz*-RP	frz operon	Forward primer: GAGTCCTGGCTTGCGCCGTT Reverse primer: CCGCTCCATCGCAGCCTGAA	Van der Westhuizen and Bragg (2012)	843
hlyF-FP *hlyF*-RP	Putative avian haemolysin	Forward primer: GGCCACAGTCGTTTAGGGTGCTTACC Reverse primer: GGCGGTTTAGGCATTCCGATACTCAG	Johnson et al. (2008)	450
iutA-FP *iutA*-RP	Aerobactinsiderophore receptor	Forward primer: GGCTGGACATCATGGGAACTGG Reverse primer: CGTCGGGAACGGGTAGAATCG	Johnson et al. (2008)	302
kpsM-FP *kpsM*-RP	Capsule-protein transport of polysaccharides	Forward primer: CAGCCTCGCGGCTTAGCTCC Reverse primer: TGCACGCGCACTGCTTGAGA	Van der Westhuizen and Bragg (2012)	335
vat-FP *vat*-RP	Vacuolating autotransporter toxin	Forward primer: CGCTTCAGGTGCGCTGACCA Reverse primer: AAGGGAGACGATGCCCGCCT	Van der Westhuizen and Bragg (2012)	498
pstB-FP *pstB*-RP	PstSCAB system	Forward primer: CGCGCTCGTCCATGTCAGCA Reverse primer: CGGAACAGCGTGCGGAAGGT	Van der Westhuizen and Bragg (2012)	198
uvrY-FP *uvrY*-RP	APEC virulence regulator	Forward primer: TGAGTGCGATTCGTTCTGTC Reverse primer: TCTCCGCATTACACAGACCA	Herren et al. (2006)	286

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Extended-spectrum β-lactamase production and antimicrobial resistance in *Klebsiella pneumoniae* and *Escherichia coli* among inpatients and outpatients of Jimma University Specialized Hospital, South-West, Ethiopia

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Extended spectrum β-lactamases (ESBLs) have emerged as a major threat worldwide with limited treatment options. The prevalence of ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* strains largely remain unknown in Ethiopia. The study was aimed at determining the occurrence of extended spectrum β-lactamase-producing *E. coli* and *K. pneumoniae* among inpatient and outpatient settings, their antimicrobial resistance profile and associated risk factors in Jimma University Specialized Hospital (JUSH). A total of 471 consecutive, non repetitive clinical specimens were collected among inpatients (n=314) and outpatients (n=157). Among these, 112 isolates of *K. pneumoniae* (n=27) and *E. coli* (n=85) were recovered. Overall prevalence of extended spectrum beta lactamase (ESBL) producers was 38.4% (n=43) of total isolates. Extended spectrum beta lactamases were found in 28.2% (n=24) of *E. coli* and 70.4% (n=19) of *K. pneumoniae*. Extended spectrum beta lactamase producers mediated very high resistance to both beta-lactams and non-betalactams, and they were significantly higher among in-patients (46.4%) than out-patients (14.3%). On Multivariate analysis, treatment with third generation cephalosporin was identified as a sole risk factor for acquisition of ESBL enzyme. Our findings confirmed that infection due to extended spectrum beta lactamase-producing *E. coli* and *K. pneumonia* is prevalent in JUSH and that exposure to third generation cephalosporin was associated with these infections. The magnitude of *E. coli* and *K. pneumoniae* infection was more in inpatients with higher levels of extended spectrum beta lactamase production than outpatients.

Key words: *Escherichia coli*, *Klebsiella pneumoniae,* extended spectrum β-lactamases, inpatients, outpatients.

INTRODUCTION

The problem of microbial drug resistance has achieved a global dimension and an alarming magnitude, being one of the leading unresolved problems in public health. The relentless evolution of resistance, in the face of a decrease in the development of new antimicrobial agents active against resistant pathogens, has led to an increa-

sing number of cases in which the pathogen is resistant to most, or even all, drugs available for clinical use (Rossolini and Mantengoli, 2008). β-Lactam agents such as penicillins, cephalosporins, monobactams and carbapenems are among the most frequently prescribed antibiotics worldwide (Pitout et al., 2005). β-Lactams account for approximately 50% of global antibiotic consumption (Livermore, 1998). Bacterial resistance to β-lactam antibiotics occurs by three mechanisms: failure of the β-lactam to reach the penicillin-binding proteins (PBPs), low-affinity binding to the PBPs and inactivation of the drug by β-lactamases (Holbrook and Lowy, 1998). Among this, β-lactamases are the commonest cause of bacterial resistance to β-lactam antimicrobial agents (Livermore, 1995).

The introduction of the third-generation cephalosporins into clinical practice in the early 1980s was heralded as a major breakthrough in the fight against β-lactamase-mediated bacterial resistance to antibiotics. The first report of plasmid-encoded β-lactamases capable of hydrolyzing the extended-spectrum cephalosporins was published in 1983 in Germany (Lautenbach et al., 2001). Later these enzymes were named extended-spectrum β-lactamases (ESBLs) (Shah et al., 2004). Since then, several outbreaks have been reported in a number of European countries and the USA, and the problem has reached endemic dimensions in several places worldwide (Giamarellou, 2005).

An extended-spectrum β-lactamase is any β-lactamase that can confer resistance to the oxyiminocephalosporins (e.g. cefotaxime, ceftriaxone, ceftazidime) and monobactams (e.g. aztreonam), but not to the cephamycins (e.g. cefoxitin and cefotetan) and carbapenems (e.g. imipenem, meropenem, and ertapenem), and which can be inhibited by β-lactamase inhibitors such as clavulanic acid (Pitout and Laupland, 2008). ESBLs are known as extended-spectrum because they are able to hydrolyze a broader spectrum of β-lactam antibiotics than the simple parent β-lactamases from which they are derived (Al-Jasser, 2006). More than 500 variants of ESBL have been described and the majority of these belong to the Temoniera (TEM), sulfhydryl variable (SHV) and Cefotaximase-Munich (CTX-M) family (http://www.lahey.org/studies/webt.htm).

K. pneumoniae and *E. coli* remain the major ESBL-producing organisms isolated worldwide, but these enzymes have also been identified in several other members of the Enterobacteriaceae family (Pitout and Laupland, 2008). ESBL-producing *E. coli* and and *K. pneumoniae* (ESBL-EK) pathogens are of great concern for many reasons. First, ESBL-EK isolates are often difficult to treat because they carry plasmids that confer resistance to multiple antibiotics. Second, patients with ESBL-EK infections may experience a delay in appropriate therapy because current methods of identification can leave them undetected. Third, patients with ESBL-EK infections have significantly longer hospital stays and incur greater hospital charges than do patients without these infections. Finally, patients with ESBL-EK infections have an increased risk of death when compared with patients with non-ESBL-EK infections (Bisson et al., 2002). A recent report from the Infectious Diseases Society of America listed ESBL-producing *Klebsiella* spp. and *E. coli* as one of the six drug-resistant microbes to which new therapies are urgently needed (Pitout and Laupland, 2008).

So far, no study has been conducted on ESBL production on both *E. coli* and *K. pneumoniae* simultaneously in Ethiopia. This study is aimed to determine prevalence and antibiotic susceptibility pattern of ESBL producing *E. coli* and *K. pneumoniae* from inpatients and outpatients that attend Jimma University Specialized Hospital. It also identifies possible risk factors for infections with ESBL producing *E. coli* and *K. pneumoniae*.

METHODS AND MATERIALS

Laboratory based comparative cross-sectional study design was conducted from September 2011 to February 2012 at Jimma University specialized hospital (JUSH), Ethiopia. The hospital is a 300 bedded teaching hospital which covers population of over 1 million. Sample size was estimated using Epi-info statistical software package (version 3.4.3, WHO Atlanta) for cross sectional studies of two population proportion to attain inpatient to outpatient ratio of 2:1. Patients' demographic data, clinical diagnoses, risk factor and specimen types were recorded for all patients included during the study period by using a questionnaire. All collected specimens were inoculated on the MacConkey agar (Oxoid, England). *E. coli* and *K. pneumoniae* was identified by their characteristic colony appearance: pink or yellow to colorless colonies (due to fermentation of lactose) from MacConkey agar, Gram-staining reaction and confirmed by the pattern of biochemical profiles using standard procedures (Koneman et al., 2006). An isolate was considered as *E. coli* when it is Indole positive, citrate negative, lysine positive, gas and acid producer, ferments mannitol, urea negative and motile. An isolate was identified as *K. pneumoniae* when it is indole negative, citrate positive, ferments mannitol, lysine positive, urea slow producing and non-motile. The

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Abbreviations: BSI, Blood stream infection; **CLSI,** Clinical and Laboratory Standards Institute; **CTX-M,** Cefotaximase-Munich; **DDST,** double disk synergy test; **ESBL,** extended spectrum β-lactamase; **ESBL-EK,** ESBL-producing *Escherichia coli* and *K. pneumoniae*; **ESBL-EC,** ESBL-producing *E. coli*; **ESBL-Kp,** ESBL-producing *K. pneumoniae*; **MDR,** multi drug resistant; **PBP,** penicillin-binding protein; **SHV,** sulphydryl variable; **TEM,** for Temoniera-name of a patient; **TMP,** trimethoprim; **SMX,** sulfamethoxazole; **WHO,** World Health Organization.

antimicrobial susceptibility was done by using Kirby-Bauer disc diffusion technique on Mueller Hinton agar (Oxoid, England) with commercially available antimicrobial discs. Strains were tested against the following antimicrobial agents: cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), amoxicillin-clavulanic acid (20/10 µg), cephalothin (30 µg), ampicillin (10 µg), carbenicillin (100 µg), trimethoprim-sulfamethoxazole (25 µg), chloramphenicol (30 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), gentamicin (10 µg), amikacin (30 µg), and tetracycline (30 µg).

All *E. coli* and *K. pneumoniae* isolates were screened for ESBL production by disk diffusion method using ceftazidime (30 µg), cefotaxime (30 µg) and ceftriaxone (30 µg) antibiotic disks (Oxoid & MAST) as recommended by Clinical and Laboratory Standards Institute (CLSI, 2005). Each disc was placed on Muller Hinton agar manually and incubated for 16-18 hours at 35°C. Isolates with reduced susceptibilities to cefotaxime (zone diameter of ≤27 mm), ceftazidime (zone diameter of ≤22 mm) and/or ceftriaxon (zone diameter of ≤25 mm) was suspected as a potential ESBL producer (Clinical and Laboratory Standard Institute, 2005). Potential ESBL producers were confirmed by double disc synergy test (DDST). A susceptibility disc containing amoxicillin-clavulanate was placed in the centre of a Mueller-Hinton agar plate, and a disc containing 30µg of ceftazidime, ceftriaxone and cefotaxime were placed 15 mm (centre to centre) from the amoxicillin-clavulanate disc. Discs were incubated at 37°C for 16-18 h. Enhancement of inhibition zone of any these of cephalosporin discs on the side facing the amoxicillin-clavulanate disc was interpreted as ESBL positive (Jarlier et al., 1988). Multidrug resistance was defined as resistance to 2 or more classes of antibiotics (quinolones, trimethoprim-sulfamethoxazole, tetracycline or aminoglycosides).

The data was processed and analyzed for descriptive statistics using SPSS statistical software, version 16.0. All variables were examined by univariate analysis using the Chi-square or Fisher's exact test, as appropriate. Multivariate analysis was performed for variables that were independently associated with ESBL-infection on univariate analysis. *P*-value less than 0.05 was considered statistically significant. The study was done after gaining a full approval from the Ethical Review Board of Jimma University and Jimma University Specialized Hospital.

RESULTS

Patient population and source of specimens

Overall, 471 patients were included in the study (314 inpatients and 157 outpatients). From these, 273 (58%) were females and 198 (42%) were males. The mean age of participants was 31.15 years (±16.97 SD). *E. coli* and *K. pneumoniae* were isolated from 112 (23.8%) clinical specimens, constituting 85 (18%) and 27 (5.7%) of total prevalence, respectively. These isolates were recovered from urine 46(40.2%), vaginal swab 25(22.3%), sputum 18(17%), pus 14(12.5%), eye discharge 6(5.4%) and blood 3(2.7%). Three-fourth (n=84) of isolates were obtained from inpatients and the remaining one-fourth (n=28) was from outpatients. ESBL producing *E. coli* and *K. pneumoniae* was detected in 43/112 (38.4%) of the isolates. The mean age of patients infected by ESBL producers was 36.79 years (±18.89 SD). The majority 31/43 (72.1%) of ESBL isolates were obtained from females and the rest 12/43 (27.9%) were isolated from males. There was no association between ESBL production and specific sex groups (p > 0.05). Nineteen (70.4%) isolates of *K. pneumoniae* was found to be positive for ESBL. ESBL production was significantly higher among *K. pneumoniae* than *E. coli* isolate (p < 0.01). The prevalence of ESBL-producing *E. coli* and *K pneumonia* was 4/157 (2.5%) in outpatients and 39/314 (12.4%) in inpatients, and thus the risk of development of ESBL-production was 5 times higher in inpatients as compared to outpatients with significant difference (p < 0.05) (Table 1).

Table 1. Distribution of ESBL-production according to isolates and settings.

	Total isolate N (%)	ESBL Producers	Non ESBL producers	*P-value*
Organism				
K. pneumoniae	27(24.1)	19(70.4)	8(11.6)	<.01
E coli	85(75.9)	24(28.2)	61(88.4)	
Department				
Inpatient	84(75)	39(46.4)	45(53.6)	.002
Out patient	28(25)	4(14.3)	24(85.7)	

Associated factors for ESBL-EK

All included variables were evaluated among inpatients and only five variables were analyzed among outpatients. On univariate analysis, prior exposure to antibiotic was the associated with ESBL-production among both hospitalized and non-hospitalized patients. Treatment with third generation cephalosporins, severity of illness, length of hospital stay and chronic heart failure (CHF) and medical ward admission were additionally associated with ESBL infection among hospitalized patients. On multivariate analysis, treatment with third generation cephalosporin (ceftriaxone) is the only risk factor associated with ESBL infection (Table 2a and b).

Antibiotic resistance profile of ESBL-EK

The *ESBL* producing *E. coli* and *K. pneumoniae* were significantly resistant to third-generation cephalosporins as compared to non-producers (p<0.05) (Table 3). Resistance conferred by ESBL producing *K. pneumoniae* and *E. coli* to ceftazidime, cefotaxime and ceftriaxone was 97.7, 100 and 100%, respectively. On the other hand, non ESBL isolates were almost susceptible to third generation cephalosporins with 91.3, 98.6 and 100% susceptibility against ceftazidime, cefotaxime and ceftriaxone, respectively. Good susceptibility was observed with amikacin in both ESBL (83.7%) and non ESBL producers ((97.1%). Both ESBL producer and non-producer isolates were completely (100%) resistant to carbenicillin.

Table 2a. Characteristics of ESBL-EK and non-ESBL-EK infected patients among outpatient settings.

Characteristic	Category	ESBL positive	ESBL negative	*P-value* (Univariate)
Out-patient variable				
Sex	Female=21	2	19	0.*212*
	Male=7	2	5	
Previous antibiotic medication	Yes=14	4	10	0.031
	No=14	0	14	
Previous hospital admission	Yes=3	1	2	0.318
	No=25	3	22	
History of ICU admission	Yes=0	0	0	
	No=28	4	24	-------
Severity of illness	Critical=8	2	6	*0.305*
	Subcritical=20	2	18	
Recent surgery	Yes=2	1	1	0.134
	No=26	3	23	

Table 2b. Characteristics of ESBL-EK and non-ESBL-EK infected patients among inpatient settings.

Characteristic	Category	ESBL positive	ESBL negative	*P-value* (Univariate)	AOR (C.I 95%)
In-patient variable					
Sex	Female=64	29	35	0.458	
	Male=20	10	10		----------
Previous antibiotic medication	Yes =60	32	28	0.045	0.350(0.11-1.111)
	No =24	7	17		
Previous hospital admission	Yes=16	7	9	0.811	
	No=68	32	36		----------
Length of hospital stay	>15 days=29	19	10	0.011	0.470(0.167-1.319)
	<15 days=55	20	35		
Treatment with 3G cephalosporins	Yes=27	21	6	<0.001	0.141(0.046-0.431)
	No=57	18	39		
History of ICU admission	Yes=5	3	2	0.530	
	No=79	36	43		----------
IV insertion	Yes=62	31	31	0.271	
	No=22	8	14		----------
Severity of illness	Critical	37	34		
	Subcritical	2	11	0.015	1.035(0.157-6.805)
Recent surgery	Yes =16	4	12	0.056	
	No=68	35	33		----------
Underlying disease	Diabetes	0	2	0.183	
	Cardiac failure	13	5	0.013	
	Hypertension	1	3	0.379	
	Hepatitis	2	0	0.124	----------
	None	23	35	1	
Type of ward	Medical=44	25	19	0.045	
	Surgical=12	7	5	0.372	
	Gynecology 20	3	17	1	----------
	Pediatrics =4	3	1	0.24	

Table 3. Comparison of the susceptibility profiles of ESBL-producing and non–ESBL-producing EK.

Antibiotic	Total isolate N (%)		ESBL-EK (n=43) N (%)		Non ESBL-E (n=69) N (%)		P- value
	R	S	R	S	R	S	
Ceftazidime	48(42.9)	64(57.1)	42(97.7)	1(2.3)	6(8.7)	63(91.3)	<0.01
Cefotaxime	44(39.3)	68(60.7)	43(100)	0	1(1.4)	68(98.6)	<0.01
Ceftriaxon	43(38.4)	69(61.6)	43(100)	0	0	69(100)	<0.01
AMC	56(50)	56(50)	38(88.4)	5(11.6)	18(26.1)	51(73.9)	<0.01
Cephalothin	97(86.6)	15(13.4)	43(100)	0	54(78.3)	15(21.7)	0.001
Gentamicin	42(37.5)	70(62.5)	36(83.7)	7(16.3)	6(8.7)	63(91.3)	<0.01
Amikacin	9(8)	103(92)	7(16.3)	36(83.7)	2(2.9)	67(97.1)	0.011
Nalidixic acid	59(52.7)	53(47.3)	36(83.7)	7(16.3)	23(33.3)	46(66.7)	<0.01
Ciprofloxacin	48(42.9)	64(57.1)	33(76.7)	10(23.3)	15(21.7)	54(78.3)	<0.01
Norfloxacin	43(38.4)	68(60.7)	29(67.4)	14(32.6)	14(20.3)	55(79.7)	<0.01
Nitrofurantoin	31(27.7)	81(72.3)	22(51.2)	21(48.8)	9(13)	60(87)	<0.01
Chloramphenicol	49(43.8)	63(56.2)	33(76.7)	10(23.3)	16(23.2)	53(76.8)	<0.01
Tetracycline	79(70.5)	33(29.5)	39(90.7)	4(9.3)	40(58)	29(42)	<0.01
TS	77(68.8)	35(31.2)	41(95.3)	2(4.7)	36(52.2)	33(47.8)	<0.01
Ampicillin	93(83)	19(17)	43(100)	0	50(72.5)	19(27.5)	<0.01
Carbenicillin	112(100)	0	43(100)	0	69(100)	0	-

AMC-amoxacillin/clavulinate, TS- trimethoprim/sulfamethoxazole.

Table 4. Resistance to specific antimicrobials in isolates from inpatients versus outpatients.

Antibiotic	Susceptibility profile n (%)				P- value
	Inpatient		Outpatient		
	R	S	R	S	
Ceftazidime	43(51.2)	41(48.8)	5(17.9)	23(82.1)	0.002
Cefotaxime	39(46.4)	45(53.6)	5(17.9)	23(82.1)	0.007
Ceftriaxon	39(46.4)	45(53.6)	4(14.3)	24(85.7)	0.003
AMC	47(56)	37(44)	9(32.1)	19(67.9)	0.029
Cephalothin	76(90.5)	8(9.5)	21(75)	7(25)	0.037
Gentamicin	38(45.2)	46(54.8)	4(14.3)	24(85.7)	0.003
Amikacin	9(10.7)	75(89.3)	0	28(100)	0.071
Nalidixic acid	49(58.3)	35(41.7)	10(35.7)	18(64.3)	0.038
Ciprofloxacin	41(48.8)	43(51.2)	7(25)	21(75)	0.027
Norfloxacin	37(44)	47(56)	6(21.4)	22(78.6)	0.033
Nitrofurantoin	28(33.3)	56(66.7)	3(10.7)	25(89.3)	0.020
Chloramphenicol	41(48.8)	43(51.2)	8(28.6)	20(71.4)	0.062
Tetracycline	64(76.2)	20(23.8)	15(53.6)	13(46.4)	0.022
TS	63(75)	21(25)	14(50)	14(50)	0.013
Ampicillin	73(86.9)	11(13.1)	20(71.4)	8(28.6)	0.059
Carbenicillin	84(100)	0	28(100)	0	___

Resistance pattern between outpatient and inpatient isolates

Generally, inpatient isolates showed higher rates of resistance to most tested antibiotics, when compared with outpatient isolates. The difference in susceptibility between inpatient and outpatient isolates was statistically significant for 12 (75%) of the 16 tested antibiotics ($p<0.05$). However, the rates of resistance to amikacin, chloramphenicol, ampicillin and carbenicillin, were not significantly different between inpatient and outpatient isolates (Table 4).

Multi drug resistant ESBL-EK

The resistance rates of ESBL isolates to 2 or more classes

Table 5. The resistance rates of ESBL isolates to 2 or more classes of non-beta lactam antibiotics.

Antibiotic combination	Resistance rate N (%)
TS and T	38 (88.4)
TS, T and NA	35 (81.4)
TS, T, NA and CN	30 (70)
TS, T, NA, CN and CIP	26 (60.5)
TS, T, NA, CN, CIP and CAF	22 (51.2)
TS, T, NA, CN, CIP, CAF and F	9 (20.9)
TS, T, NA, CN, CIP, CAF, F and Ak	3 (7)

T- Tetracyclin, TS- trimethoprim-sufamethoxazole, NA- nalidixic acid, CN- gentamicin, CIP- ciprofloxacin, C- chloramphenicol, F- nitrofurantoin, Ak- amikacin.

classes of antibiotics are given in Table 5, descending from the lowest to the highest resistant isolates. ESBL-EK generally showed higher rates of resistance to antibiotics tested than non-producers. About 88.4% of ESBL isolate were multi drug resistant exhibiting cross-resistance against both cotrimoxazole and tetracycline. Resistance to three non beta-lactam antibiotics was observed among 35 (81.4%) isolates; in addition approximately 70% of ESBL positive isolates were cross-resistant to four non beta-lactam antibiotics (tetracycline, cotrimoxazole, nalidixic acid and gentamicin). The coexistence of ESBL phenotypes with five, six and seven types of non beta-lactam antibiotics were 26 (60.5%), 22 (51.2%) and 9 (20.9%) respectively. Three (7%) ESBL isolates were completely resistant to all panels of antibiotics tested.

DISCUSSION

ESBLs are widespread all over the world. The prevalence and genotype of ESBLs from clinical isolates vary according to the country and even hospital at which they are isolated from (Kim et al., 2010). The overall prevalence of ESBLs in the current study was 38.4% (43/112). This frequency is higher than continental surveys conducted in Europe (11%), South America (18.1%), North America (7.5%) and Asia-Pacific (14.2%) regions (Hawser et al., 2011; Turner, 2005). The higher prevalence seen in our study as compared to developed countries might be explained by the fact that developed countries have strict infection control policies and practices, shorter average hospital stays, better nursing barriers that are known to substantially decrease the chances of acquisition and spread of ESBL producing strains.

On the other hand, the prevalence of ESBL observed in this study is lower than that of a study done in Tanzania (45.2%) conducted variably on urinary isolates (Moyo et al., 2010). The decline observed in our study can be attributed to the inclusion of various types of specimens. Regardless of such myriad variation, this finding agrees with previous reports on ESBL production done in United Arab Emirates (Al-Zarouni et al., 2008).

Although *E. coli* ranks higher in the number of infection occurrences than *K. pneumoniae*, the predominant ESBL producer in our setting is *K. pneumoniae*. ESBL production was significantly higher among *K. pneumoniae* than *E. coli* ($p<0.01$). This finding is in agreement with previous report done among *K. pneumoniae* and *E. coli* with respective prevalence of 70 and 28% in Pakistan, and 51.5 and 39.1% in Tanzania, which demonstrated predominance of ESBL production by *K. pneumoniae* than *E. coli* (Shah et al., 2003; Moyo et al., 2010). Other study had also demonstrated conquest of ESBL producing *K. pneumoniae* not only over *E. coli* but also over other group of Gram negative bacilli including the family *Enterobacteriaceae* (Galas et al., 2008). The predilection of ESBL production by *K. pneumoniae* has never been clearly explained (Mshana et al., 2009). Our observation that *K. pneumoniae* was significantly associated with ESBL production merely reflects local and worldwide epidemiology which clearly shows that ESBL production has been more frequently observed in these bacteria than in *E. coli*.

Undesirable turn of events transpired when ESBL producing *E. coli* were detected in the community. Three (75%) of the four ESBL producers from outpatients were *E. coli.* The occurrence of ESBL-producing *E. coli* isolates in the community is in keeping with the global trend of emergence of community-acquired infections caused by ESBL-producing strains, in particular those which harbor the CTX-M gene. These gene have been reported in Africa (Kariuki et al., 2007). A recent report from Japan showed that patients with fecal carriers of ESBL-producing *E. coli* contributed substantially to urinary tract infections (Niki et al., 2011). This tendency could markedly change the approach to the treatment of urinary tract infections and as well as other infections due to ESBL producing *E. coli* that are encountered in the outpatient setting.

The interesting point of the present study was a correlation between multiple antibacterial resistances and ESBL positive phenotypes. This finding indicates that ESBL-producing strains of *K pneumoniae* and *E. coli* are more likely to have diminished susceptibility to non-β-lactam antibiotics when compared with non-ESBL-producing isolates, further curtailing the number of drugs useful against these bacteria. This result has been confirmed by others (Moyo et al., 2010; Mshana et al., 2009). This is mainly associated with unique property of the large ESBL plasmid which is capable of incorporating and subsequently coding for resistant determinants to non beta-lactam antimicrobial agents (Jacoby and Sutton, 1991). Thus our study results well support the fact that ESBL producers not only confer high levels of resistance to third generation cephalosporins but also to non-beta lactams like aminoglycosides, fluoroquinolones, tetracyclines and cotrimoxazole.

Thirty-eight (88.8%) of ESBL isolates showed multi drug resistance from 2 to 8 types of non beta lactam antibiotics tested. Of particular concern is that three (7%) of ESBL producing isolates were resistant to all panels of antibiotics used. Thus, the presence of an ESBL is a good marker of the MDR phenotype. In the present study, amikacin has retained good susceptibility rates due to its absence of use as empirical therapy and nonexistence of considerable cross-resistance with third generation cephalosporins. Similarly, study from Egypt also showed the high percentage of susceptibility to amikacin among antibiotics tested (Zaki, 2007). These findings have significant implication for empirical management of patients infected with ESBL organisms using amikacin.

Third-generation cephalosporin specifically ceftriaxone is one of the most commonly used classes of antibiotics for hospitalized patients in Ethiopia, as observed during this study, exerting predominant selective pressure for the emergence of resistance among pathogenic microorganisms. On multivariable analysis, use of third generation cephalosporins was identified as the only risk factors significantly associated with infection due to ESBL producers. This finding is in accordance with previous studies disclosing that indiscriminate use of third-generation cephalosporins was related to the selection of ESBL-producing organisms (Lautenbach et al., 2001). Use of cephalosporins is not only associated with ESBL infection, but also it was found to be a risk factor for colonization with ESBL producing organisms (Levy et al., 2010). As a result, the higher percentage of ESBL-producing *E. coli* or *K. pneumoniae* in the current study may be due to the greater selective pressure imposed by extensive use of third-generation cephalosporins. This association has been best displayed by interventional study which demonstrated decline in the prevalence of ESBL-EK colonization from 7.9 to 5.7% following restriction of third-generation cephalosporins (Bisson et al., 2002). In general, the association of ESBL with third-generation cephalosporins suggests that the best way to control these pathogens in our hospital is to reduce the use of these antibiotics.

ESBLs occurrence was significantly higher among isolates from inpatients than outpatients [39 (46.4%) vs. 4(14.3%)] (P =0.002). Nosocomial acquisition of ESBL producing *E. coli* and *K. pneumoniae* bacteremia has been reported indicating that hospital environment played a crucial role in maintenance of ESBL producing organism (Kang et al., 2006). Furthermore higher rate of fecal carriage of ESBL-producing organisms among inpatients (26.1%) than among outpatients (15.4%) is documented elsewhere in Saudi Arabia (Kader et al., 2007). This suggests that nosocomial acquired organisms are more likely to become ESBL producer.

More than 70% of strains isolated from both inpatient and outpatient groups showed resistance to ampicillin, cephalothin and carbenicillin. This may alarm the presence of the classic beta lactamase which was recognized among this isolates prior to isolation of ESBL enzymes (Livermore, 1995). In addition, marked resistance to tetracycline and co-trimoxazole was observed in the inpatient group (77.4% to tetracycline and 75% to TMP-SMZ) and with slight decrease in the outpatient group (51.7% to tetracycline and 48.3% to TMP-SMZ), this may be explained by the frequent use of both antibiotics in the community as well as in our hospital. Therefore, the use of this drug is questionable in suspected *E. coli* and *K. pneumoniae* infection in our setting.

Limitation

We are familiar with the limitation of study, as noted in all observational studies. Molecular epidemiological study and characterization of ESBL types were not conducted. Second, we did not assess certain clinical features such as ICU admission and urinary catheterization as potential risk factor for infection with ESBL producing EK due to little number of cases which are insignificant number to be included during study period. Third, our study was conducted in Jimma University Specialized Hospital, and the results may not be generalizable with other institutions.

Conclusion

Our data provide evidence that the ESBL is prevalent in Jimma University Specialized Hospital. Majority of ESBL producing strains are from inpatients and only few are community isolates. Therefore, it is very urgent to address the problem of hospital acquired infections caused by ESBL-producing bacteria. Use of third generation cephalosporin was the only independent predictor of ESBL-producing *E. coli* or *K. pneumoniae* infection. These agents should not be used in infections due to confirmed ESBL producers because resistance to third-generation cephalosporin is often accompanied by resistance to fluoroquinolones, aminoglycosides, TMP-SMX and tetracyclines.

Conflict of interest

The author(s) have not declared any conflict of interests.

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REFERENCES

Al-Jasser AM (2006). Extended-Spectrum Beta-Lactamases (ESBLs). A Global Problem. Kuwait Med J. 38(3): 171-185.

Al-Zarouni M, Senok A, Rashid F, Al-Jesmi SM, Panigrahi D (2008). Prevalence and antimicrobial susceptibility pattern of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in the United Arab Emirates. Med. Princ. Pract.17: 32-36.

Bisson G, Fishman NO, Edelstein PH, Lautenbach E (2002). Extended-spectrum β-lactamase–producing *Escherichia coli* and *Klebsiella* species: risk factors for colonization and impact of antimicrobial formulary interventions on colonization prevalence. Infect. Control Hosp. Epidemiol. 23: 254-260.

Clinical and Laboratory Standard Institute WP (CLSI) (2005). Clinical and Laboratory Standard Methods. Performance standards for antimicrobial susceptibility testing: seventeenth informational supplement. M100-S115.

Galas M, Decousser JW, Breton N, Godard T, Allouch PY, Pina P, Collège de Bactériologie Virologie Hygiène (ColBVH) Study Group (2008). Nationwide Study of the Prevalence, Characteristics, and Molecular Epidemiology of Extended-Spectrum-β-Lactamase-Producing *Enterobacteriaceae* in France. Antimicrob. Agents Chemother. 52(2): 786–789.

Giamarellou H (2005). Multidrug resistance in Gram-negative bacteria that produce extended-spectrum β-lactamases (ESBLs). Clin. Microbiol. Infect. 11(Suppl. 4): 1–16.

Hawser SP, Bouchillon SK, Lascols C, Hackel M, Hoban DJ, Badal RE, Cantón R (2011). Susceptibility of European *Escherichia coli* clinical isolates from intra-abdominal infections, extended-spectrum b-lactamase occurrence, resistance distribution, and molecular characterization of ertapenem-resistant isolates (SMART 2008–2009). Clin. Microbiol. Infect. 18(3):253-9.

Holbrook KA, Lowy FD (1998). β-Lactam Antibiotics. Cancer Invest. 16: 405-412.

Jacoby GA, Sutton L (1991). Properties of plasmids responsible for production of extended-spectrum beta-lactamases. Antimicrob. Agents Chemother. *35:* 164-169.

Jarlier V, Nicolas MH, Fournier G, Philippon A (1988). Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactama gents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 10: 867-878.

Kader AA, Kumar A, Kamath KA (2007). Fecal carriage of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in patients and asymptomatic healthy individuals. Infect. Control Hosp. Epidemiol. 28: 1114-1116.

Kang CI, Kim SH, Bang JW, Kim HB, Kim NJ, Kim EC et al. (2006). Community-Acquired versus Nosocomial *Klebsiella pneumoniae* Bacteremia: Clinical Features, Treatment Outcomes, and Clinical Implication of Antimicrobial Resistance. J. Korean Med. Sci. 21: 816-822.

Kariuki S, Revathi G, Corkill J, Kiiru J, Mwituria J, Mirza N, Hart CA (2007). *Escherichia coli* from community-acquired urinary tract infections resistant to fluoroquinolones and extended-spectrum beta-lactams. J. Infect. Dev. Ctries. 1(3): 257-262.

Kim MH, Lee HJ, Park KS, Suh JT (2010). Molecular Characteristics of Extended Spectrum β-Lactamases in *Escherichia coli* and *Klebsiella pneumoniae* and the Prevalence of qnr in Extended Spectrum β-Lactamase Isolates in a Tertiary Care Hospital in Korea. Yonsei Med. J. 51(5): 768-774.

Koneman EW, Allen SD, Janda WM, Win WC (2006). The *Enterobacteriaceae.* In: Color Atlas and Text Book of Diagnostic Microbiology. 6th ed. 211-302.

Lautenbach E, Patel JB, Bilker WB, Edelstein PH, Fishman NO (2001). Extended-spectrum b-lactamase–producing *Escherichia coli* and *Klebsiella pneumoniae*: risk factors for infection and impact of resistance on outcomes. Clin. Infect. Dis. 32: 1162–1171.

Levy SSS, Mello MJG., Gusmao-filho FAR, Correia JB (2010). Colonisation by extended-spectrum b-lactamase-producing *Klebsiella spp*. in a paediatric intensive care unit. J. Hosp. Infect. 76: 66-69.

Livermore DM (1995). β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. 8: 557–584.

Livermore DM (1998). β-lactamase-mediated resistance and opportunities for its control. J. Antimicrob. Chemother. 41(Suppl): 25–41.

Moyo SJ, Aboud S, Kasubi M, Lyamuya EF, Maselle SY (2010). Antimicrobial resistance among producers and non-producers of extended spectrum beta lactamases in urinary isolates at a tertiary Hospital in Tanzania. BMC Res. Notes 3:348.

Mshana SE, Kamugisha E, Mirambo M, Chakraborty T, Lyamuya EF (2009). Prevalence of multiresistant gram-negative organisms in a tertiary hospital in Mwanza, Tanzania. BMC Res. Notes 2:49.

Niki M, Hirai I, Yoshinaga A, Ulzii-Orshikh L, Nakata A, Yamamoto A, Yamamoto M, Yamamoto Y (2011). Extended-spectrum b-lactamase-producing *Escherichia col*i strains in the feces of carriers contribute substantially to urinary tract infections in these patients. Infection 39(5):467-471.

Pitout JDD, Laupland KB (2008). Extended-spectrum beta-lactamase-producing *Enterobacteriaceae*: an emerging public health concern. Lancet Infect. Dis. 8:159-166.

Pitout JDD, Nordmann P, Laupland KB, Poirel L (2005). Emergence of *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) in the community. J. Antimicrob. Chemother. 56: 52–59.

Rossolini GM, Mantengoli E (2008). Antimicrobial resistance in Europe and its potential impact on empirical therapy. Clin. Microbiol. Infect. 14 (Suppl. 6): 2–8.

Shah AA, Hasan F, Ahmed S, Hameed A (2003). Prevalence of extended-spectrum beta-lactamases in nosocomial and outpatients (ambulatory). Pak. J. Med. Sci. 19: 187-191.

Shah AA, Hasan F, Ahmed S, Hameed A (2004). Extended-Spectrum β-Lactamases (ESBLs): Characterization, Epidemiology and Detection. Crit. Rev. Microb. 30(1): 25–32.

Turner PJ (2005). Extended-Spectrum β-Lactamases. Clin. Infect. Dis. 41: S273-S275.

Zaki MS (2007). Extended Spectrum β-Lactamases among Gram-negative bacteria from an Egyptian pediatric hospital: a two-year experience. J. Infect. Dev. Ctries. 1(3): 269-274.

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Full Length Research Paper

Regeneration of cumin (*Cuminum cyminum* L.) plants from callus and establishment of dual culture of host and parasite (*Alternaria burnsii*)

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Tissue culture technique can be used for normal callus culture and regeneration of a new clone. Nowadays the technique is used for establishment of dual culture of host and parasite, and regeneration of disease free plants. Normal callus was established and maintained on MS-medium supplemented with 6-benzylaminopurine (BAP, 1.0 mgl^{-1}) and naphthalene acetic acid (NAA, 4.0 mgl^{-1}). Efficient shoot bud and regeneration of cumin plant from callus was observed on combination of Kinetin (0.5 mgl^{-1}) + indole-3-acetic acid (IAA, 1.0 mgl^{-1}), BAP (0.1 mgl^{-1}) + NAA (1.0 mgl^{-1}) with 25 mgl^{-1} adenine sulphate (AS). Dual culture of *Alternaria burnsii* on cumin (*Cuminum cyminum*) callus was established on MS medium by using infected seeds. Dual culture was developed on MS-medium supplemented with NAA (4.0 mgl^{-1}), BAP (1.0 mgl^{-1}), biotin (1.0 mgl^{-1}), thiamine hydrochloride (1.0 mgl^{-1}), ascorbic acid (25.0 mgl^{-1}) and casein hydrolysate (1.0 mgl^{-1}). The dual culture developed from the infected seeds which were surface sterilized, indicates that the blight disease is basically seed borne in nature. Casein hydrolysate (1.0 mgl^{-1}) and ascorbic acid (25 mgl^{-1}) was found to be best for the growth of the fungus in the dual culture. It was concluded that the study can be used for disease free plants and enhance the growth of the fungus through control the nutrients from the host during disease development.

Key words: Cumin, *Alternaria burnsii,* callus, dual culture.

INTRODUCTION

Cumin (*Cuminum cyminum* L.) is one of the important seed spices and highly remunerative cash crop which is extensively grown in Rajasthan state of India. The seeds are used as a condiment or spice in the various preparations like vegetables, curries, pickles, etc. Cumin seeds also have medicinal properties. Cumin water locally called

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Abbreviations: Kn, Kinetin; **BAP,** 6-benzylaminopurine; **NAA,** naphthalene acetic acid; **IAA,** indole-3-acetic acid; **IBA,** indole butyric acid; **2-4D,** 2,4-dichlorophenoxy acetic acid; **MS,** Murashige and Skoog medium.

Jaljeera has very good cooling effect. Its seeds are also being used in the treatment of gonorrhoea, chronic diarrhoea and dyspepsia. A number of fungal bacterial and mycoplasma-like organisms (MLOs) diseases occurred in cumin crop and influenced the production negatively. Among the diseases, the heavy losses are sustained due to attack of *Alternaria*blight. *In vitro* culture of plant tissues provides an excellent system for the study of growth, metabolism and morphogenesis. In absolutely controlled conditions callus or cell suspension cultures can be raised from almost every part of a plant starting from shoot apex, axillary bud, leaf mesophyll cells, epidermis, pith, cambium, phloem, root, flower parts and fruit parts. The culture techniques thus gradually advanced using new information of growth regulators.

Somatic embryogenesis in some species of Apiaceae family has been discussed by Lourdes and Alfermann (1994) especially in cumin (Soorni et al., 2012). Dual cultures have been used frequently in plant pathological studies for growth of biotrophic fungi and *in vitro* expression of disease resistance (Goyal, 1990; Chorabik, 2014). The expression of resistance of the host to fungi in tissue culture has been investigated using combination of *Phytophthora infestans* and *Solanum tuberosum* (Ingram and Robertson, 1965). Tissue culture techniques are now being used widely as tool in crop improvement by generation of novel disease resistant lines of crop plants (Garg et al*.,* 1986). Plants resistant to different diseases have been regenerated from susceptible parent in several crops (Larkin and Scrowcroft, 1981; Thanutony et al*.,* 1983). The technique also provides opportunities to evaluation of resistance against *Albugo candida* and *Alternaria brassica* in *Brassica juncea* (Sharma and Singh, 1995). Debnath et al*.* (2006) studied *in vitro* selection of Ridomil tolerant strains of *Brassica juncea* var. *varuna* and developed resistant cell line against white rust disease through tissue culture technique. Kiran et al*.* (2003) studied the effect of cultural filtrate of *A. brassicae* on biochemical constituents of calli of Brassicas.

In this experiment the tissue culture technique was used to study the isolation and establishment of normal callus culture, regeneration of cumin plant from callus and dual culture of host and parasite.

MATERIALS AND METHODS

Isolation and establishment of normal callus cultures and regeneration

For normal callus cultures and regeneration, the certified seeds of *Cuminum cyminum* variety RZ-19 were surface sterilized with 0.1% mercuric chloride for 3-4 min and rinsed 3-4 times with distilled water. The seeds were then germinated on water-agar medium under aseptic conditions. These cultures were kept at 2000 lux intensity light at 25±2°C. The seeds germinated within 10-12 days. The hypocotyl was cut into small pieces using a sterilized scalper and transferred to 100 ml "Erlenmeyer" flask containing 40.0 ml of solidified MS-medium (Murashige and Skoog, 1962). The stock solutions containing basal salts for the media were stored under refrigeration. Small quantities of stocks were prepared as per the need to avoid old stocks. The growth regulators that is auxins and cytokinins were used in basal MS-medium at various concentrations. For the auxins *viz*., indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), indole butyric acid (IBA) and 2,4-dichlorophenoxy acetic acid (2,4-D), stocks were prepared by dissolving them in a few drops of absolute alcohol initially. Cytokinins *viz*., 6-furfuryl amino purine (kinetin) and 6-benzylaminopurine (BAP) were initially dissolved in a few drops of HCl (1 N). Final volume of all the growth regulators was made up by adding distilled water. The pH of the medium was always adjusted to 5.8 by adding 1 N NaOH or 1 N HCl.

Hypocotyal derived callus was sub-cultured on MS medium supplemented with cytokinins (BAP/Kinetin/Zeatin 0.1-3.0 mg l^{-1}), auxins (IAA/NAA/BAP 0.5-5.0 mg l^{-1}) and adenine sulphate (15-55 mgl^{-1}) added singly and in combination. All the manipulations were done aseptically under the laminar air flow bench; pre-sterilized with ultraviolet light for forty minutes. Inoculation and transfer of explants was carried out in sterilized conditions. The cultures were incubated in culture chamber at 26±2°C and 55% relative humidity in 2000 lux intensity light and 16 h photoperiod.

Dual culture of host and parasite

The callus which was derived from the hypocotyl of cumin variety RZ-19 which is susceptible to blight, was sub-cultured aseptically on MS-medium supplemented with NAA (4.0 mgl^{-1}), BAP (1.0 mgl^{-1}), ascorbic acid (5-40 mgl^{-1}) and different concentrations of biotin, thiamine hydrochloride and casein hydrolysate ranging from 0.05 mg l^{-1} to 5.0 mg l^{-1}. The cultures were incubated in culture chamber at 30±2°C with 65% relative humidity in complete darkness. In some of the replicates, fungus appeared on the callus. The fungus from the dual culture was examined microscopically at different time intervals during its growth period. It was stained in cotton blue and mounted in lactophenol. The slides were photographed with Nikon's Alpha photo-trinocular microscope.

Statistical analysis

The statistical analysis of the data was carried out using Duncan's multiple range test (DMRT) at the $P < 0.05$ level of probability to test the differences between the treatment means using SPSS software. All the data on shoot length and root length were analysed using one-way ANOVA.

RESULTS AND DISCUSSION

Callus induction

Normal callus initiated from hypocotyl was cultured on MS medium with different concentrations of auxins *viz*. 2, 4-D, NAA, IAA and IBA (1.0-5.0 mg l^{-1} each) alone or in combination with cytokinins, that is BAP or kinetin (1.0-5.0 mg l^{-1}) (Table 1). Best growth of the callus was observed on NAA (4.0 mg l^{-1}) in combination with BAP (1.0 mgl^{-1}). The callus produced was healthy, soft, fast growing and light green in colour. This callus showed fast growth after further sub-culturing. NAA along with all tried concentrations of kinetin did not give good response (Table 2). On these combinations the growth of callus was poor and callus turned brown. Similar results have also been observed by Yadav (2003), Shukla et al*.* (1996) and Soorni et al*.* (2012) in *Cuminum cyminum*.

Table 1. Callus induction in hypocotyal explants of *Cuminum cyminum* inoculated on MS supplemented with different concentration of various growth hormones.

Medium:	MS + sucrose (30%) + auxins viz., 2,4-D, NAA, IAA (1.0-5.0 mgl^{-1})
Incubation:	At 26±2°C and 16 h photoperiod (2000 lux) upto 4 weeks.
Inoculum:	Hypocotyl of germinated seeds.

Auxin/s concentration (mgl^{-1})		**Response**	**Remarks**
Control	**MS without auxin**	**Nil**	**Nil**
2,4-D	1.0	+	Callus was fast growing, fragile and whitish in colour.
	2.0	+++	
	3.0	+++	
	4.0	+++	
	5.0	+++	
IAA	1.0	+	Callus was healthy soft fast growing and yellowish green in colour
	2.0	++	
	3.0	++	
	4.0	+++	
	5.0	+++	
IBA	1.0	–	Callus was poor in growth and brownish in colour.
	2.0	–	
	3.0	+	
	4.0	++	
	5.0	+++	

– = No callus; + = Moderate callus ; ++ = Good callus; +++ = profuse callus.

Regeneration and complete plant formation

Efficient shoot morphogenesis (>20%) was observed in callus cultures and sub-cultures with the combination of kinetin (0.5 mgl^{-1}) + IAA (1.0 mgl^{-1}), BAP (0.1 mgl^{-1}) + NAA (1.0 mgl^{-1}) with 25 mgl^{-1} adenine sulphate (AS). The reproducibility of this morphogenesis was very high (Tables 3 and 4). Callus only proliferated further, getting a semi-compact nature when ratio of the cytokinin to axuin was high and remained parenchymatous on high auxin to cytokinin ratio.

Multiple shoots induced at above mentioned media when separated and sub-cultured on MS medium supplemented with 0.1 -1.0 mgl^{-1} IAA + 0.3-1.0 mgl^{-1} Kinetin. Maximum roots were observed at the base on 0.3 mgl^{-1} kinetin +0.1 mgl^{-1} IAA and complete plant was obtained. These plants grew well under *in-vitro* conditions and flowered profusely but seed setting was not observed (Figure 1). Regenerated plants were put to hardening for transplant into soil. For hardening, healthy plantlets were transferred to plastic cups filled with sterilized soil rite. Plantlets were covered with moist polythene bags and kept in environmental chamber maintained at 25±2°C. Inside the environmental chamber the lightening was provided with florescent light for 14 h a day. The plantlets were irrigated with MS salt solution at 1/4 strength. However, the plantlets survived to a maximum of 10 days. Valizadeh et al. (2007) observed that the B_5 medium containing 2 mgl^{-1} NAA and 2 mgl^{-1} Kinetin was the best treatment for callus and root induction and regeneration simultaneously. Kahrizi and Soorni (2013) reported that 0.1 mg/l NAA plus 0.4 mg/l BAP and 0.1 mg/l NAA plus 1 mg/l BAP combinations were determined as the highest level for indirect shoot regeneration (with 25.83 % and 25 %, respectively).

Dual culture of host and parasite

Some fungal growth was observed on callus after 15-20

Table 2. Effect of different plant growth regulators on callus growth of calli induced on MS medium containing NAA (4.0 mgl $^{-1}$).

Medium	MS + sucrose (3.0%) + NAA (4.0 mgl $^{-1}$) + BAP/Kinetin (1.0-5.0 mgl $^{-1}$)		
Incubation	**At 26±2°C and 16 h photoperiod (2000 lux) upto 4 weeks**		
Inoculum	**Hypocotyl of germinated seeds**		
BAP	1.0	+++	Callus was healthy, soft, fast growing on further sub culturing and light green in colour.
	2.0	++	
	3.0	++	
	4.0	+	
	5.0	+	
Kinetin	1.0	++	Callus was poor in growth and brown in colour.
	2.0	+	
	3.0	–	
	4.0	–	
	5.0	–	

– = No callus; + = Moderate callus ; ++ = Good callus; +++ = profuse callus

Table 3. Shoot morphogenesis in callus cultures of cumin on various concentrations of growth regulators and Adenine sulphate.

BAP/Kinetin (mgl^{-1})	Adenine sulphate (mgl^{-1})	IAA/NAA (mgl^{-1})					
		0.5	1.0	2.0	3.0	5.0	Shoot length (cm)
0.1	15	+	++	-	-	-	1.90^{a}
0.5	25	+	++	-	-	-	1.12^{b}
1.0	35	-	+	-	-	-	1.30^{b}
2.0	45	-	-	-	-	-	-
3.0	55	-	-	-	-	-	-

- = Callus proliferation; + = Poor shoot morphogenesis (< 15 %); ++ = Good shoot morphogenesis (>20 %).Means with the same letter (superscript) in the columns showing shoot length do not significantly differ (P = 0.05) based on Duncan Multiple Range Test.

days of inoculation as brownish black mycelium (Table 5 and Figure 2A and B). The callus was brown in colour. Later on growth of callus was retarded and the fungus covered most of its surface. The callus did not grow further. Results showed that biotin enhanced the growth of fungus in culture at all concentrations. 1.0 mg l-^{1} biotin was found to be optimum for fungal growth. However, on increasing biotin concentration the growth of callus and fungus was found to be poor. Ascorbic acid along with biotin (1.0 mg l^{-1}) enhanced the growth of fungus in dual culture and 25.0 mg l^{-1} ascorbic acid was found optimal. Absence of ascorbic acid in the medium and its higher concentrations were found inhibitory for the growth of fungus. Casein hydrolysate 1.0 mg l^{-1} along with thiamine hydrochloride (1.0 mg l^{-1}), ascorbic acid (25 mg l^{-1}) and biotin (1.0 mg l^{-1}) were found to be the best for growth of fungus in dual culture. The technique can be used for development of disease free plant and it is very useful in plant pathologist to develop a disease free clones. Similarly, use of interactions in dual cultures *in vitro* to evaluate the pathogenicity of fungi and susceptibility of host plant genotypes were also reported by Chorabik (2013) in different perennial plants.

Morphological studies of the fungus from diseased callus

Microscopic examination after 15-20 days of culture revealed that fungal hyphae proliferated freely over the callus surface. The conidia were formed at the tip of conidiophores. Conidiophores were septate, light olive in colour and gave rise to chains of conidia (Figure 2C). Diseased callus cultures were raised on combination of NAA (4.0 mgl^{-1}), BAP (1.0 mgl^{-1}), ascorbic acid (25.0 mgl^{-1}), biotin (1.0 mgl^{-1}), thiamine hydrochloride (1.0 mgl^{-1}) and casein hydrolysate (1.0 mgl^{-1}) on MS-medium and resulted in good growth of fungus (*Alternaria burnsii*) on the callus. Earlier, Goyal (1990) raised diseased callus culture from infected inflorescence axis explant

Table 4. Effect of combinations of Cytokinins (Kinetin) and Auxins (IAA/NAA) on Shoot and root length of *Cuminum cyminum* var. RZ-19.

Growth regulators(mgl $^{-1}$)	Shoot length(cm)	Root length(cm)	Remarks
BAP + IAA (mgl^{-1})			
0.3 + 0.1	3.7^{efg}	2.0^{ab}	
0.3 + 1.0	3.8^{defg}	2.1^{a}	
1.0 + 0.1	3.0^{h}	1.9^{abc}	
1.0 + 1.0	3.1^{h}	1.7^{cde}	
BAP + NAA (mgl^{-1})			
0.3 + 0.1	3.9^{cdef}	1.8^{bcd}	
0.3 + 1.0	4.0^{bcde}	2.1^{a}	
1.0 + 0.1	3.5^{g}	1.7^{cde}	
1.0 + 1.0	4.1^{abcd}	1.9^{abc}	
Kinetin + IAA (mgl^{-1})			
0.3 + 0.1	4.3^{ab}	2.0^{ab}	
0.3 + 1.0	4.4^{a}	1.6^{de}	Best media for root initiation and shoot initiation
1.0 + 0.1	3.6^{fg}	2.1^{a}	
1.0 + 1.0	3.7^{efg}	1.5^{e}	
Kinetin + NAA (mgl^{-1})			
0.3 + 0.1	4.2^{abc}	1.7^{cde}	
0.3 + 1.0	4.4^{a}	1.9^{abc}	
1.0 + 0.1	4.0^{bcde}	1.8^{bcd}	
1.0 + 1.0	3.9^{cdef}	2.0^{ab}	

Means with the same letter (superscript) in the columns showing shoot length and root length do not significantly differ (P = 0.05) based on Duncan Multiple Range Test.

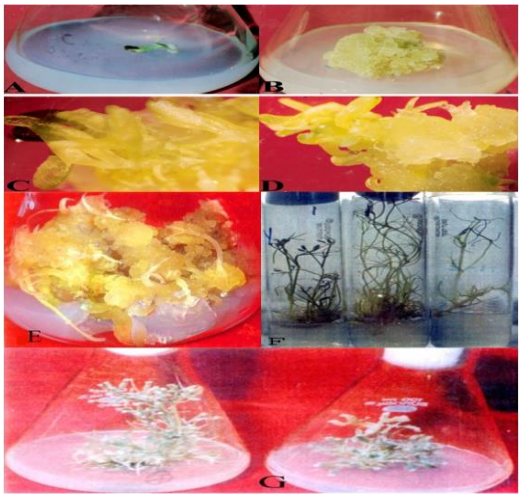

Figure 1. A. Callus induction from hypocotyls on MS+NAA (4.0 mgl^{-1}) and BAP (1.0 mgl^{-1}). B. Stock callus. C and D. Shoot bud initiation on MS + Kinetin (0.5 mgl^{-1}) + IAA (1.0 mgl^{-1}) +BAP (0.1 mgl^{-1}) + NAA (1.0 mgl^{-1}) + AS (25 mgl^{-1}). E. Shoot initiation. F. Shoot elongation on MS + Kinetin (0.5 mgl^{-1}) + IAA (1.0 mgl^{-1}) + BAP (0.1 mgl^{-1}) + NAA (1.0 mgl^{-1}) + AS (25 mgl^{-1}) and root induction on MS + 0.3 mgl^{-1} Kinetin + 0.1 mgl^{-1} IAA. G. Complete plant formation.

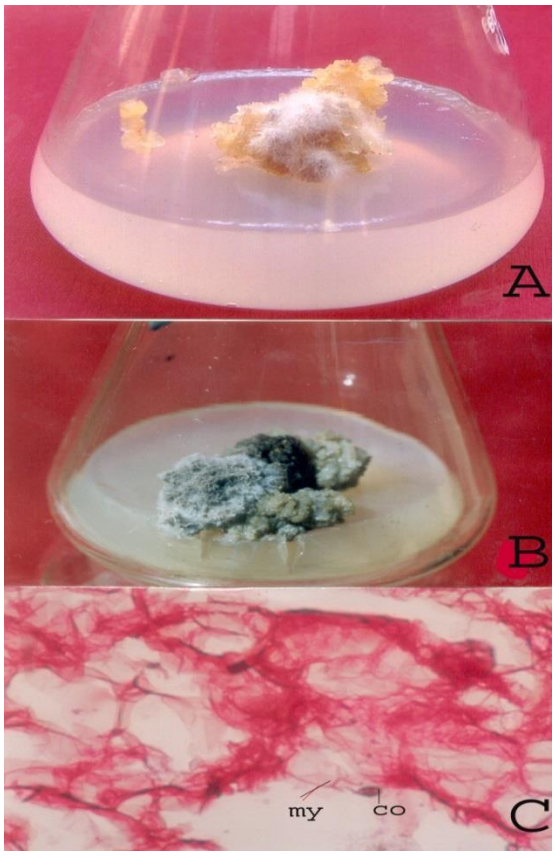

Figure 2. A. Initiation of dual culture on MS-medium + NAA (4 mgl^{-1}) + BAP 1.0 mgl^{-1}) + Biotin (1.0 mgl^{-1}) + Thiamine hydrochloride (1.0 mgl^{-1}) + Casein hydrolysate (1.0 mgl^{-1}) + Ascorbic acid (25 mgl^{-1}). **B.** 30 days old dual culture. **C.** Microphotograph of cells of dual culture showing mycelium and conidia.

Table 5. Effect of biotin, ascorbic acid, thiamin hydrochloride and casein hydrolysate on dual culture of *Cuminum cyminum* and *Alternaria burnsii* on modified MS-medium supplemented with NAA (4 mgl^{-1}) and BAP (1.0 mgl^{-1}).

Ascorbic acid (mgl^{-1})	Biotin (mgl^{-1})				Thiamin hydrochloride (mgl^{-1})				Casein hydrolysate				Growth characteristics of fungus
	0.5	1.0	2.0	3.0	0.5	1.0	2.0	3.0	0.5	1.0	2.0	3.0	
0	-	-	-	-	-	-	-	-	-	-	-	-	No fungal growth
5	+	+	+	-	-	-	-	-	-	-	-	-	Poor growth
10	-	-	-	-	-	-	-	-	-	-	-	-	No fungal growth
15	+	++	+	+	+	+	-	-	+	+	-	-	Poor fungal growth
20	++	++	++	-	-	+	+	-	+	++	-	-	Moderate fungal growth
25	++	+++	++	-	++	+++	++	-	++	+++	++	-	Good fungal growth
30	-	++	+	+	-	++	-	-	-	++	-	-	Poor fungal growth
35	-	-	-	-	-	-	-	-	-	-	-	-	No fungal growth
40	-	-	-	-	-	-	-	-	-	-	-	-	No fungal growth

– = No response; + = poor fungal growth; ++ = moderate fungal growth; +++ = good fungal growth.

(*Brassica juncea* and *Albugo candida*) on MS-medium supplemented with IBA (10.0 mgl^{-1}) and kinetin (0.5 mgl^{-1}).

Plant tissues which contain higher concentrations of this ascorbic acid are more resistant to pathogens

(Plazek, 2011).

Conflict of interest

The author(s) have not declared any conflict of interests.

REFERENCES

Chorabik KN (2013). The use of interactions in dual cultures in vitro to evaluate the pathogenicity of fungi and susceptibility of host plant genotypes. Biochemistry, Genetics and Molecular Biology "Environmental Biotechnology-New Approaches and Prospective Applications", book edited by Marian Petre, ISBN 978-953-51-0972-3, and Published under CC BY 3.0 license.

Chorabik KN (2014). Interactions between embryogenic callus of *Abies alba* and *Heterobasidion* spp. in dual cultures. Biol. Plant. 58(2):363-369.

Debnath M, Sharma SL, Kant U (2006). *In vitro* selection for fungicides tolerance in mustard and regeneration of white rust resistant cell line. Phytomorphology 56(1&2): 85-91.

Garg GK, Singh US, Khetrapal RK, Kumar J (1986). Application of tissue culture in plant pathology. In: Experimental and conceptual plant pathology, oxford & IBH Publishing Co. Pvt. Ltd, 83-120.

Goyal BK (1990). Studies on fungal induced abnormalities of *Brassica juncea* L. Crenz. & Coss *in vivo* and *in vitro*. Ph.D. Thesis, University of Rajasthan, Jaipur, India.

Ingram DS, Robertson (1965). Interaction between *Phytopthora infestans* and tissue culture of *Solanum tuberosum.* J. Gen. Microbiol. 40:431-437.

Kahrizi D, Soorni J (2013). Study on shoot regeneration and somatic embryogenesis in cumin (*Cuminum cyminum* L.) landraces. Biharean Biologist 7(1):37-41.

Kiran D HR, Mehta N, Sangwan MS (2003). Effect of culture filtrate of *Alternaria brassicae* on biochemical constituents of calli of brassicas. J. Mycol. Pl. Pathol. 33(1):51-55.

Larkin PJ, Scroweroft WR (1981). Somaclonal variation a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60:197-214.

Lourdes BC, Alfermann AW (1994). Somatic embryogenesis in some Apiaceae species. In: Abstr. 8th Asian Symposium on Medicinal Plants and Spices National Production, June 12-16 Meloka, Malaysia.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Płażek A (2011). *Patofizjologiaroślin*. Patophysiology of plants. Publishing House of the University of Agriculture in Cracow. 1:139.

Sharma TR, Singh BM (1995). Generation and evaluation of somaclones of *Brassica juncea* for resistance to *Albugo candida* and *Alternaria brassicae.* Proc. Ind. Natl. Sci. Acad. Part B. Biol. Sci. 61(2):155-161.

Shukla MR, Subhash N, Patel DR, Patel SR (1996). *In vitro* studies in cumin (*Cuminum cyminum* L.). In : Biotechnology of spices, medicinal and aromatic plants (Eds.) Edision S, Ramana KV, Sasikumar B, Nirmal Babu and Snathosh. J Eapen, Ind. Soc. Spices. Calicut. pp. 45-48.

Soorni J, Kahrizi D, Molsaghi M (2012). The effect of photoperiod and 2,4-D concentrations on callus induction of *Cuminum cyminum* leaf explants: An Important medicinal plant. Asian J. Boil. Sci. 5(7):378-383.

Thanutony D, Furusawa I, Vamamoto M (1983). Resistant tobacco plants from protoplasts derived callus selected for their resistance to *Pseudomonas* and *Alternaria* toxins. Theor. Appl. Genet. 66:209-216.

Valizadeh M, Kazemi SK, Nematzadeh GA (2007). Effect of plant growth regulators on callus induction and regeneration of cumin (*Cuminum cyminum*). Ind. J. Sci. 2(2):25-30.

Yadav P (2003). *In vivo* and *in vitro* studies of some important fungal diseases of cumin - an economically important spice. Ph.D. Thesis University of Rajasthan, Jaipur.

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