



Effect of various antibiotics against the *Actinobacillus lignieresii*, *Acinetobacter* and *Citrobacter* species isolated from the frozen semen of cattle

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Received 8<sup>th</sup> June 2015 and Revised 25<sup>th</sup> September 2015

**Abstract:** Antibiogram evaluation of *Actinobacillus lignieresii*, *Acinetobacter* and *Citrobacter* species were isolated from frozen semen of cattle. Different antibiotics such as amikacin, ofloxacin, neomycin, cephalixin, amoxicillin, ampicillin, gentamicin, sulphamethoxazole/trimethoprim, and erythromycin were tested against the isolated bacterial species. Amikacin, neomycin and gentamicin were highly effective against *Acinetobacter*. While the organism exhibited high resistance against the amoxicillin, erythromycin and cephalixin. Ofloxacin, amikacin, sulphamethoxazole and gentamicin were more effective against the *Actinobacillus lignieresii* than the neomycin and amoxicillin. Amikacin, neomycin, ofloxacin and gentamicin were highly effective against the *Citrobacter* species. Erythromycin, amoxicillin and cephalixin did not show response against the organism. Amikacin was found highly effective against the *Acinetobacter*, *Actinobacillus lignieresii* and *Citrobacter* species were isolated from frozen bovine semen.

**Key words:** Semen, bovine, extender, antibiotics, contaminants, bacteria

## 1. INTRODUCTION

Artificial insemination (AI) has been successful technique that is practiced for the breeding of cattle and other domestic animal species around the globe. The method is a valuable tool that benefiting breeders to gain high quality genetic potential from proven bulls (Funk, 2006; Verkerk, 2003). Generally, semen is packaged in straws approximately 0.25 ml or 0.5 ml, pellets and flattened plastic bags for freezing and storage. The frozen straws and flattened plastic bags are transported in liquid nitrogen for the artificial insemination (Bwanga *et al.*, 1991; Weitze *et al.*, 1991). However, there is a risk of contamination of semen from pathogens during the packaging and storage can deteriorate quality of semen and reproductive efficiency (Russell *et al.*, 1997).

Normally, fresh semen or every ejaculate contains some of nonpathogenic microorganisms that are not adversely affecting the semen quality. However, the excessive load or harmful microorganisms may result in infertile mating (Thacker *et al.*, 1984). The semen may get contaminated with pathogenic and non-pathogenic microbial agents during processing and storage of the semen. These microbes gain access to the semen and can transfer of the serious diseases in recipient farm animals. This may lead to bacteraemia, viraemia and local infections in different parts of genital tract (Diemer *et al.*, 1996; Thibier and Guerin, 2000). Hence, the successful breeding program depends on quality of semen produced at semen production centers.

It is prerequisite of the semen production centers to ensure the produced semen should be free from microorganisms from environment and donor bulls. Several studies reported the presence of bacterial organisms such as *Staphylococcus aureus*, *Coxiella burnetti*, *Acinetobacter cacloaceticus*, *Escherichia coli*, *Brucella suis*, *Pantoeau agglomerans*, *Histophilus somni*, *Enterobacter cloacae*, *Staphylococcus sciuri*, *Ureaplasma diversum*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Enterobacter-coccus*, *Chlamydophila abortus Micrococcus*, *Leptospira*, *Corynebacteriu* and *Flavobacterium* species in the frozen semen of farm animals (Bielanski *et al.*, 2003; Corona and Cherchi, 2009; D'Angelo *et al.*, 2006; Hobson *et al.*, 2013; Kruszewska and Tylewska-Wierzbanska, 1997; Ramaswamy *et al.*, 1990; Ramaswamy *et al.*, 1994; Ramaswamy *et al.*, 2002; Schlafer and Miller, 2007; Thibier and Guerin, 2000; Vinodh *et al.*, 2008). In addition, bacterial load may show variation in different breeds (Sannat *et al.*, 2015).

Routinely, antibiotics are added to semen extender in order to control bacterial contaminants and to improve the fertility in artificial insemination in cattle (de Jarnette *et al.*, 2004). In general, combination of streptomycin and penicillin are added to diluents for bovine semen (Andrabi *et al.*, 2001; Sansone *et al.*, 2000). The combination of lincospectin, gentamycin and tylosin added in bovine semen extender is quite effective for controlling in microbes such as; *Campylobacter fetus Mycoplasma* and *Pseudomonas*

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species (Guerin and Thibier, 1993). In contrast, the combination of lincomycin, gentamicin, spectinomycin and tylosin (LGST) were added in bull semen extender to check the efficiency of this combination of the antibiotics against mycoplasma species. The combination failed to control growth of mycoplasma species in the semen extender contained LGST antibiotic mixture. This indicated the various antibiotics used in semen extender are not necessary to control infection or microbial contamination (Visser *et al.*, 1999). Traditionally, addition of penicillin in semen extender is not responding against various microorganisms such as mycobacteria, corynebacteria, vibrio, hemophilus brucella, ureaplasmas and mycoplasmas (Shin *et al.*, 1988).

Antibiotic resistance is an emerging problem for various pathogens contaminates bovine semen used for artificial insemination in local conditions. Therefore, this study is designed to evaluate the efficacy of various antibiotics against the bacterial isolates identified from bovine semen used for artificial insemination.

## 2. MATERIALS AND METHODS

One hundred frozen semen samples of cattle were collected under sterile hygienic condition from the local semen production centers. The semen samples were contained in straws and sterilized bijou bottles in artificial insemination kits, which contained liquid nitrogen and brought to the laboratory.

Different dehydrated media were used for the culture or presence of any bacteria in the frozen semen samples. Dehydrated nutrient agar (Difco, 2000), MacConkey agar (Difco, 2000) and blood agar (Difco, 2000) were rehydrated according to recommendation of manufacturer. The media were stirred to dissolve and then autoclaved at 121°C under 15 lb pressure for 15 min. Cooled and blood agar at 45-50°C was added with 5% defibrinated aseptically sheep blood. The samples were inoculated by streaking method on nutrient, blood and MacConkey's agar media and incubated aerobically at 37°C for 24 h for the presence of microbial agents. The bacterial colonies that were grown on the media were sub-cultured to achieve pure culture of bacteria. The single colony was taken for the preparation of smear and routine staining procedure. The cultured bacteria were observed for morphological characteristic.

Further the colonies were taken for the pure culture and for the biochemical properties and sugar fermentation tests. Different biochemical tests such as catalase, coagulase test, gelatin liquefaction, aesculin test, bile tolerance test, Hugh and Leifson's test, indole production test, methyl red, methyl blue Proskauer test

oxidase, triple sugar iron agar, Simmon's citrate, urease production test, nitrate reduction and sugar fermentation tests were performed as prescribed by (Abro *et al.*, 2009; Christensen *et al.*, 2002; Khalil and Gabbar, 1992). These biochemical and sugar fermentation tests were performed for the identification and confirmation of the isolates contained in the frozen semen samples.

Antibiotic sensitivity provides an answer to the suitable choice of drug for the treatment of bacterial infection. The antibiotic sensitivity was performed as described by Bauer *et al.* (1996). The different antibiotics; amikacin, ofloxacin, neomycin, chloromphenicol, cephalexin, amoxicillin, kanamycin, ampicillin, gentamicin, sulphamethoxazole/trimethoprim, tetracycline and erythromycin were used during the study. The surface of Muller Hinton agar (Difco) was dried by incubating at 37°C for 15 minutes. Bulks of pure culture colonies were suspended evenly in 2-4 sterile normal saline solution in order to match barium chloride standard for antibiotic sensitivity. A sterile cotton swab was dipped into the suspended and culture was smeared on the surface of Muller Hinton agar in such a way that all agar surfaces was covered evenly with the bacterial suspension, then incubated at 37°C for 15 minutes for plate to dry. The desired antibiotic disc were kept on agar surface with disc dispenser and lightly pressed with sterile forceps to make it adhere to surface. The plate was closed, wrapped in polythene bag, inverted (medium and disc upward) and incubated overnight at 37°C. The zone of inhibition was observed as a clear area, free from growth around the disc and a clear zone of inhibition made against organism. The zone of inhibition by the antibiotics was recorded in millimeter from the center of disc to zone. The antibiotic sensitivity was classified into highly, quite, moderately, weakly sensitive and resistant depending on the antibiotics, its contents in the disc and size of zone with the following annotations;

|  |      |
|--|------|
| Absence of clear zone around antibiotic discs            | -    |
| Clear zone with 1-2mm diameter around antibiotic discs   | +    |
| Clear zone with 2-5mm diameter around antibiotic discs   | ++   |
| Clear zone with 5-10mm diameter around antibiotic discs  | +++  |
| Clear zone with 10-15mm diameter around antibiotic discs | ++++ |

## 3. RESULTS AND DISCUSSION

In this study, one hundred frozen semen samples of cattle were collected from local semen production units and these samples were examined for the contamination or presence of bacterial species. The findings revealed that frozen bovine semen samples were found positive for *Actinobacillus lignieresii*, *Acinetobacter* and *Citrobacter* species. These bacterial species isolated from the frozen bovine semen were tested for the efficacy of various antibiotics.

*Acinetobacter* was highly sensitive to amikacin, neomycin and gentamycin and their sensitivity against the species recorded 87.5%, 84.37 and 75% respectively (Table 1). Also the organism was found quite sensitive ofloxacin and the action against the organism recorded 40.62%. The organism was weakly sensitive (9.37%) to ampicillin. The MICs of neomycin, neticilmicin, gentamycin, tobramycin, kanamycin and amikacin of the organism 8 ug/ml, 32 ug/ml, 128 ug/ml, 128 ug/ml, 64 ug/ml, 64 u/ml respectively (Xiao *et al.*, 2015). Previously, it was investigated that streptomycin and methicillin with minimum inhibition concentration (MIC) of 0.10-0.65 ug/ml effective to *Acinetobacter* species (Egwu *et al.*, 1994). *Acinetobacter baumannii* showed susceptibility to amikacin and spectinomycin and relative resistance to neomycin, kanamycin, neomycin and gentamycin (Ndegwa *et al.*, 2010). In this study, the organism was found completely resistant against amoxicillin, erythromycin and cephalixin. *Acinetobacter* species were conferring resistance against amoxicillin, erythromycin and cephalixin. The organism had shown resistance against the amoxicillin, chloromphenicol, levofloxacin, sulfadiazine and minocycline (Vamuri *et al.*, 2015). Amoxicillin was found to exhibit reduced bioactivity against the *Acinetobacter* species (Rasool *et al.*, 2015). In this study, *Actinobacillus lignieresii* was found highly sensitive to ofloxacin, amikacin, sulphamethoxazole and gentamycin and its susceptibility against the antibiotics was recorded as 68.18%, 63.63%, 56.81 and 54.54%

respectively (Table 1). In this study, the tested antibiotics for the sensitivity against the organism isolated from frozen semen of cattle are in accordance with previous report (Koral *et al* 1994). The organism was isolated from exudate from cattle conferring resistance against the antibiotics such as; cephalixin, amikacin, pencillin, erythromycin, ampicilin, cefotaxime, enrofloxacin, neomycin, amoxicillin-clavulamic acid, rifampicin, vancomycin, erythromycin and cloxacillin. Whereas, the organism was susceptible to gentamicin, tilmicosin, norfloxacin, streptomycin, deoxycycline, oxitetracycline, deoxycycline, tetracycline, phlorphenicol, cloranphenicol, ofloxacin, cefaclor, trimethprim/sulphamethoxazole, cephaclor, cephalotin, ofloxacin, nalidixic acid, polymyxin, kanamycin, fosfomycin, and nalidixic acid (Carlos *et al.*, 2013). The organism was observed quite sensitive to neomycin and amoxicillin and their sensitivity recorded as 45.45% and 29.54%.

Multidrug resistance is an emerging problem among the *Citrobacter* species and getting more attention due to nosocomial pathogen from the environment. The antibiotic resistance were shown higher against the commonly used antibiotics such as ampicillin, fluroquinolones, amikacin, gentamicin, cephalosporins (Dhanya and Bhat, 2015). *Citrobacter* species was found highly sensitive to amikacin, neomycin, ofloxacin and gentamycin and their sensitivity against the organism were recorded

Table 1. Antibiotic sensitivity against the bacterial species isolated from bovine frozen semen.

| Bacterial species                 | Antibiotic disc                 | Inhibitory zone (mm) | Sensitivity (%) | Sensitivity Induction | Degree of sensitivity |
|-----------------------------------|---------------------------------|----------------------|-----------------|-----------------------|-----------------------|
| <i>Acinetobacter</i>              | Gentamicin                      | 12                   | 75              | ++++                  | Highly sensitive      |
|                                   | Neomycin                        | 13.5                 | 84.37           | ++++                  | Highly sensitive      |
|                                   | Amikacin                        | 14                   | 87.5            | ++++                  | Highly sensitive      |
|                                   | Amoxycillin                     | 0                    | 0               | -                     | Not sensitive         |
|                                   | Erythromycin                    | 0                    | 0               | -                     | Not sensitive         |
|                                   | Cephalexin                      | 0                    | 0               | -                     | Not sensitive         |
|                                   | Ampicillin                      | 1.5                  | 9.37            | +                     | Weakly sensitive      |
|                                   | Ofloxacin,                      | 6.5                  | 40.62           | +++                   | Quite sensitive       |
| <i>Actinobacillus lignieresii</i> | Neomycin                        | 10                   | 45.45           | +++                   | Quite sensitive       |
|                                   | Ofloxacin                       | 15                   | 68.18           | ++++                  | Highly sensitive      |
|                                   | Amikacin                        | 14                   | 63.63           | ++++                  | Highly sensitive      |
|                                   | Gentamicin                      | 12                   | 54.54           | ++++                  | Highly sensitive      |
|                                   | Ampicillin                      | 0                    | 0               | -                     | Not sensitive         |
|                                   | Amoxycillin                     | 6.5                  | 29.5            | +++                   | Quite sensitive       |
|                                   | Erythromycin                    | 0                    | 0               | -                     | Not sensitive         |
|                                   | Sulphamethoxazole/Trimethoprim, | 12.5                 | 56.81           | ++++                  | Highly sensitive      |
| <i>Citrobacter</i>                | Ampicillin                      | 2.5                  | 13.8            | ++                    | Moderately sensitive  |
|                                   | Neomycin                        | 14.5                 | 80.55           | ++++                  | Highly sensitive      |
|                                   | Gentamicin                      | 11.5                 | 63.88           | ++++                  | Highly sensitive      |
|                                   | Amikacin                        | 16                   | 88.88           | ++++                  | Highly sensitive      |
|                                   | Ofloxacin                       | 12.5                 | 69.44           | ++++                  | Highly sensitive      |
|                                   | Erythromycin                    | 0                    | 0               | -                     | Not sensitive         |
|                                   | Amoxycillin                     | 0                    | 0               | -                     | Not sensitive         |
|                                   | Cephalexin                      | 0                    | 0               | -                     | Not sensitive         |

as 88.88%, 80.55%, 69.44% and 63.88% (**Table 1**) respectively. The organism was partially sensitive to ampicillin and its sensitivity was recorded 13.88%. The results in this study regarding the susceptibility of the organism to amikacin is in accordance with previous reports (Patil and Lakshmi, 2000; Shah *et al.*, 2002). The resistance to gentamycin was 63.88% and the findings closely related 52.5% (Patil and Lakshmi, 2000). The susceptibility of various antibiotics was investigated against *Citrobacter* species. It was recorded that the organism was highly susceptible to pipemidic (87.5%) and cefuroxime (68.75%). However, cefoperazone (50%) and erythromycin (37.5%) were moderately sensitive to *Citrobacter* species (Sajida, 2000). The organism was found completely resistant against the erythromycin, amoxicillin and cephalixin respectively. Our findings regarding the ampicillin are in complete agreement with previous report (Dhanya and Bhat, 2015), they observed 85% of *Citrobacter* strains were resistant to ampicillin.

#### 4. CONCLUSION

In summary, *Acinetobacter* was highly susceptible to amikacin, neomycin and gentamycin and completely resistant to amoxicillin, erythromycin and cephalixin. *Actinobacillus lignieresii* was found highly sensitive to ofloxacin, amikacin, sulphamethoxazole and gentamycin. *Citrobacter* species were shown high susceptibility against the amikacin, neomycin, ofloxacin and gentamycin and conferring the resistance to erythromycin, amoxicillin and cephalixin.

#### 5. ACKNOWLEDGEMENT

The Central Veterinary Diagnostic Laboratory (CVDL) Tandojam, Sindh, Pakistan, is highly acknowledged for providing platform and necessary facilities for the research. The authors are grateful to Dr. Parkash Dewani, CVDL, Tandojam, Sindh, Pakistan for his help in the research work.

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