MOLECULAR CHARACTERIZATION, ANTIBIOTIC SUSCEPTIBILITY AND BIOFILM FORMING ABILITY OF *PASTEURELLA MULTOCIDA* ISOLATED FROM THE RESPIRATORY MICROBIOTA OF HEALTHY BOVINES

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ABSTRACT: Livestock plays an important role in the economy of the country because it provides food producing animals. *Pasteurella multocida* which is an opportunistic pathogen of upper respiratory tract of animals and it has been known to cause many harmful diseases may survive in animal body by forming biofilm in different organs. So, the presence of this bacterium in animals may lead to transfer and threaten the human health by food chain. In the last few years, one of the major problems that alarms and evokes a major horrific threat to human being is the bacterial resistance to antimicrobial agents. Due to deficiency of antimicrobial agents in pipeline and emergence of new mechanisms of intra antimicrobial resistance, many surveillance programs have been set up for its containment. Many factors are responsible for this grave situation like high use of antibiotics in animals. So, the current study concluded that all of the 76 isolates of *Pasteurella multocida* out of 100 samples were confirmed through PCR and found sensitive to florfenicol (100%), gentamycin (92.11%), chloramphenicol (84.21%) and kanamycin (72.37%) respectively. For tetracycline and neomycin sensitivity were (69.73%) and (64.47%) respectively against the bacteria. Whereas tilmicosin and spectinomycin sensitivity were recorded as (30.27%) and (23.69%). Streptomycin (19.73%), sulfadiazine (7.89%), erythromycin (3.94%) and clindamycin (0.0 %) were least sensitive. It was also concluded that when all pure isolates of *Pasteurella multocida* were tested for Biofilm assay out of 76 isolates categorized as: non biofilm producer (7.89%), weak biofilm producer (34.21%), moderate biofilm producer (46.05%) and strong biofilm producer (11.84%). Hence, it was concluded that Pasteurella multocida isolated from cattle and buffaloes were found to form biofilm in in-vitro condition.

Keywords: Bovines, Biofilm, Antibiotic, *Pasteurella multocida,* Molecular characterization.

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INTRODUCTION

Pasteurella multocida, a Gram-negative short rod shaped bacteria which is causative agent of *Hemorrhagic septicemia* (HS) (Called GUL GHOTOO) in local language which have great importance *(Vaid et al., 2012). T*he most common bacterial agent is *Pasteurella multocida* from cases of bovine respiratory disease and pneumonia in cattle. From the feedlot cattle's the most common disease is bovine respiratory disease. The maximum cases of HS are reported in wet season especially started from December to February (Farooq *et al.,* 2007). *Pasteurella multocida* has been isolated wide-reaching from mammals and birds in corporation with various disease conditions. *Pasteurella multocida* subsp. multocida has been reported to have a broad host spectrum compared to *Pasteurella multocida* subsp. gallicida, most commonly isolated from birds, and *Pasteurella multocida* subsp. septica, isolated mostly from cats and dogs (Christensen *et al.* 2004).

Pasteurella multocida isolates are related with many clinical manifestations ranging from asymptomatic or mild chronic upper respiratory inflammation to acute, pneumonic and disseminated disease. Whereas less common *Pasteurella multocida* can also cause human infections through animal bites and scratches (Wilson and Ho, 2013). There are many virulence factors contributing to the pathogenesis of *Pasteurella multocida*. The two main surface components i.e. capsule and lipopoly saccharides (LPS) form the main typing basis of *Pasteurella multocida* (Peng *et al.,* 2018). It is recorded that the infection of *Pasteurella multocida* isolates exhibits host predilection (Wilkie *et al.,* 2012). A *Pasteurella multocida* B: 2 strain can kill cattle and buffaloes at a very low dose but it has no harmful effect on chickens even at very high doses (Aktories *et al.,*

2012). The molecular and multi-locus sequence typing method have been also designed for *Pasteurella multocida* identification and also used in epidemiology for surveillance recording (Hotchkiss *et al.,* 2011).

Pasteurella multocida has 5 capsular (A, B, D, E, F) and 16 somatic serovars. *Pasteurella* is a gram negative, capsular and cocco-bacillus which is responsible for fetal and mortal diseases in cattle and buffaloes. In poultry industry serovars 1 and 3 are mostly found whereas serovars 2 and 5 belonging to the *Hemorrhagic septicemia* are present in cattle and buffaloes (Harper *et al.* 2011). *Pasteurella multocida* B: 2 is Asian serotype whereas E: 2 is African serotype of the bacteria which causes *Hemorrhagic septicemia* (HS) in cattle, buffaloes and sometimes other species also. Moreover, *Hemorrhagic septicemia* B: 2 serotypes may exist in Africa, and E: 2 serotypes may be occurring in Asia. *Hemorrhagic septicemia* is recognized as a most conspicuous transmittable disease with high financial importance (Shivachandra *et al.,* 2011).

MATERIALS AND METHODS

Sample Collection: A total of 100 samples (nasal + tracheal swabs) were collected from bovines brought in the PAMCO (Punjab Agriculture and Meat Company) slaughter house. Samples were collected by nasal swabbing of an animal and after slaughtering a part of trachea of the same animal were collected too. For nasal swabbing a six inch sterile cotton swab was inserted into the nostril. The swabs were then placed into a sterile tube with one milliliter of sterile phosphate buffered saline (PBS). Similarly tracheal swab samples of same animal were preserved in the PBS (Gilmore *et al*. 1993). After collection, swab samples were labeled properly. Swab samples were transported to Department of Microbiology, UVAS Lahore.

Isolation of *Pasteurella multocida:* All swab samples were processed to isolate *Pasteurella multocida* and for this purpose blood agar was used which is an enriched media for the growth of Pasteurella multocida.

Culturing and Identification of *Pasteurella multocida:* Presumptive isolates from each sample were further identified using colony morphology. Swabbing was done on blood agar plates which were then incubated at 37ºC for 24 hours (Ashraf *et al*., 2011).

Identification of *Pasteurella multocida*: The microscopic characters of *Pasteurella multocida* were studied by using Giemsa staining technique (Fig 1).

Molecular identification of *Pasteurella multocida* by PCR: All the isolates were identified by using PCR. Firstly, total genomic DNA was isolated from all the isolates. Genomic DNA was isolated from overnight broth culture of *Pasteurella multocida* by using “Qiagen” DNA extraction kit. Method provided by the manufacturer was followed. This DNA was saved in -20°C to use as template for PCR reactions (Fig 2).

PCR Based Confirmation: All isolates were subjected for PCR based analysis for confirmation of *Pasteurella multocida* B: 2 serotype specifically. Previously designed Primer pairs were used in this study. The primer pair KMT1T7 and KMT1SP6 was used to identify all *Pasteurella multocida* isolates (Fig 3).

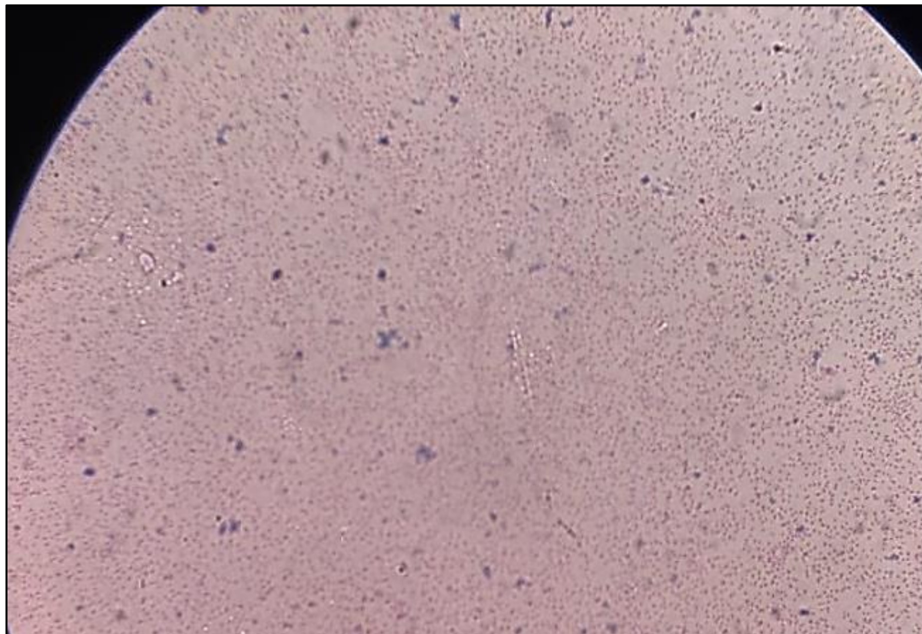

Fig 1. Morphology of *Pasteurella multocida* under 100X magnification

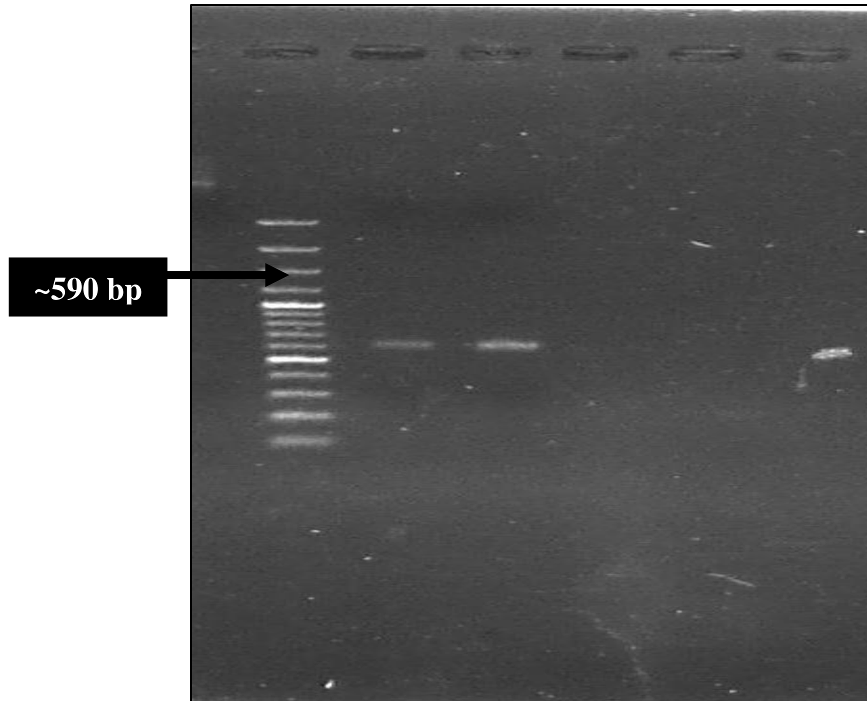

Fig 2. *Pasteurella multocida* specific PCR products (~590 bp).

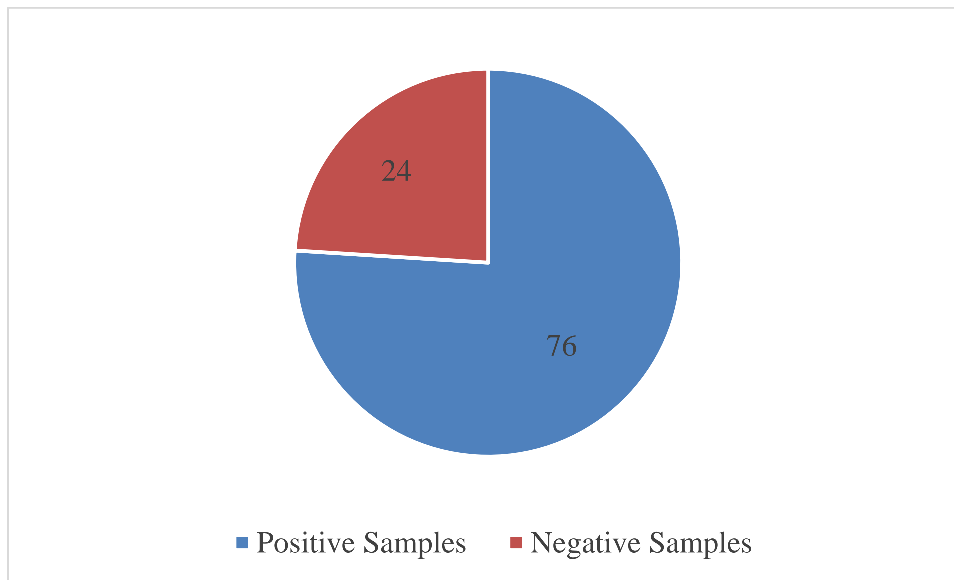

Figure 3. Number of positive samples of *Pasteurella multocida* detected through PCR

Agarose Gel Electrophoresis (AGE) to visualize DNA: AGE was performed to confirm the extraction of genomic DNA. 2% Agarose gel was composed in working solution of 1X TAE Buffer by making it thin from 50 X TAE Buffer stock solutions. Mini Horizontal Gel Apparatus was used. 1g Agarose powder was weigh and included into a 100 ml conical flask. 50ml of 1X TAE buffer was put on to the flask. Agarose was dissolved completely in microwave by heating the solution for several short intervals until the solution became clear. Solution was given time to cool down by agitating the flask intermittently to cool properly. When the solution was cooled to about 50˚C-55˚C, 3µl ethidium bromide was added in it and mixed well. Periphery of gel casting tray was locked and comb was inserted in it. Then the agarose solution was poured into the casting tray and permitted it to cool until it became solid and appeared milky white. Comb was taken out and the sealing was removed. Gel was then placed in the gel electrophoresis chamber. 1X TAE buffer was added to enough volume in

the chamber such that there was 2-3mm of its quantity is over the gel. Gel was properly immersed in buffer. Colorful product (6 µl) was directly loaded into the well and (2 µl) ladder of 1kb was also loaded in one well of the gel for the assessment of size of the amplicons. DNA bands on the gel were visualized using Cleaver Scientific Ltd gel documentation system. Gel picture was captured and labeled the wells with proper names.

Both the negative and positive terminals of tank were properly connected to power supply unit of electrophoresis apparatus (Cleaver Scientific). Negative electrodes were towards the side where DNA samples were loaded. Samples were electrophoresed at 4v/cm for 1hour. Onward after 1 hour the power supply was turned off. The tray was detached using blue nitrile disposable gloves and the gel was examined in UV trans-illuminator and then results were documented by the aid of gel documentation system (Cleaver Scientific). Gel picture was captured and labeled the wells with names.

Antimicrobial susceptibility testing: The test of antimicrobial susceptibility of all molecularly confirmed isolates was performed as per guidelines given by Clinical and Laboratory Standards Institute (CLISI) (Clinical and Institute 2017). Multiple antibiotics were tested to detect the antibiotic resistance in *Pasteurella multocida.* The antimicrobial discs used for sensitivity testing were given in the Fig 4.

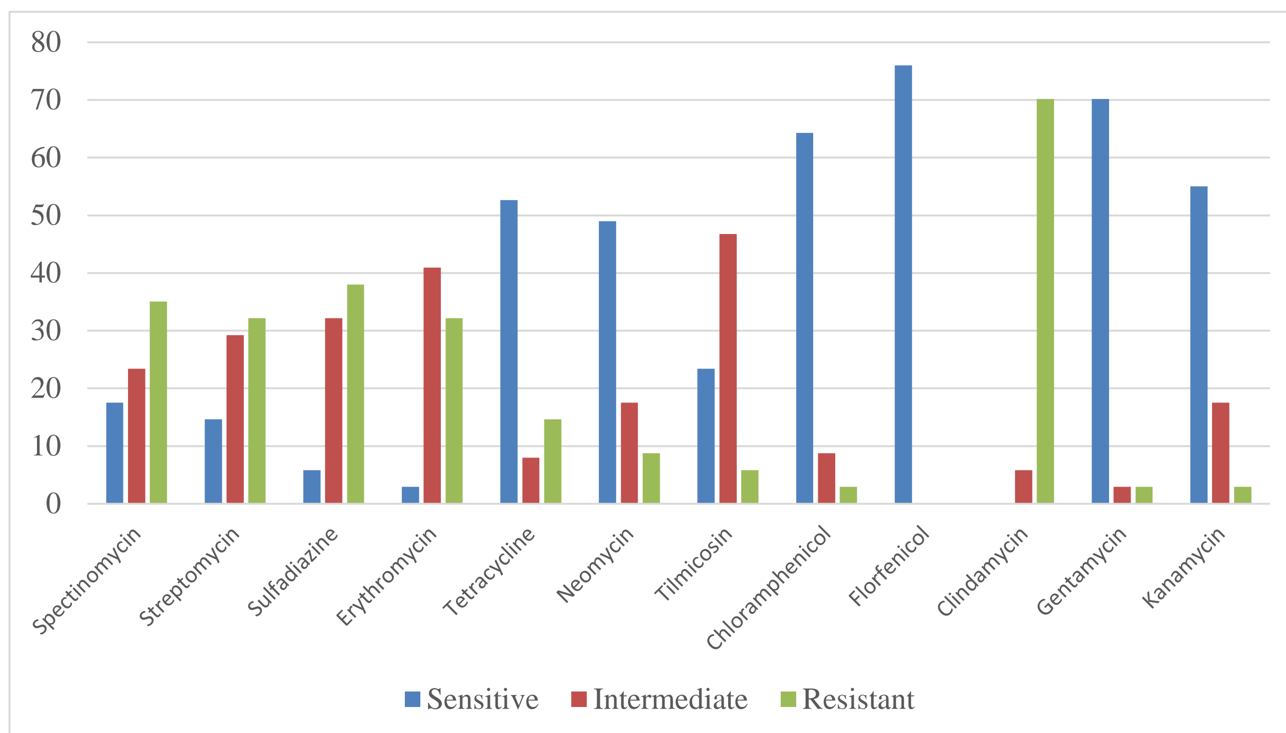

Figure 4: Antibiotic susceptibility graph of 76 isolates of *Pasteurella multocida*

Inoculum preparation and swabbing: 5 ml Normal saline (0.89%) was prepared in glass test tubes and tubes were sterilized. Purified cultures of *Pasteurella multocida* were added in sterile tubes of normal saline with the help of sterile loop. The saline tube was mixed well to create a smooth bacterial suspension. The normal saline tube holding colonies were compared with 0.5 McFarland standard tubes and cell density of normal saline containing *Pasteurella multocida* was visually adjusted according to 0.5 McFarland turbidity standards. Swab was dipped in this suspension applied onto Mueller Hinton agar plate with 5% sheep blood (Gharibi *et al.*, 2017).

Application of antimicrobial discs: A forcep was taken and sterilized with a sterile alcohol pad and was air dried. The lid of the inoculated plate was removed partially and this sterile forcep was used to dispense all antimicrobial disks on inoculated plate one at a time. Disks were gently pressed with the same forcep to ensure the complete attachment of the antimicrobial disk onto the agar surface. Six different antibiotic discs as recommended by CLSI 2017 were used. All the plates were incubated for 24 hours at 37ºC (Fig 5).

Reading of plates: All plates were examined after incubation of one day and visible clear zones of inhibition were observed. Size of zones of inhibition was then interpreted by referring to CLSI guidelines and isolated strains were considered as either sensitive (S), resistant (R) or intermediate (I).

Inoculum Preparation for Biofilm Assay: *Pasteurella multocida* was grown in 5 ml of Brain Heart Infusion (BHI) broth and incubated at 37ºC for 18 hour. McFarland standard (0.5) was adjusted for broth culture and its 20 µl was inoculated into to 2 ml of freshly prepared medium in an eppendorf tube.

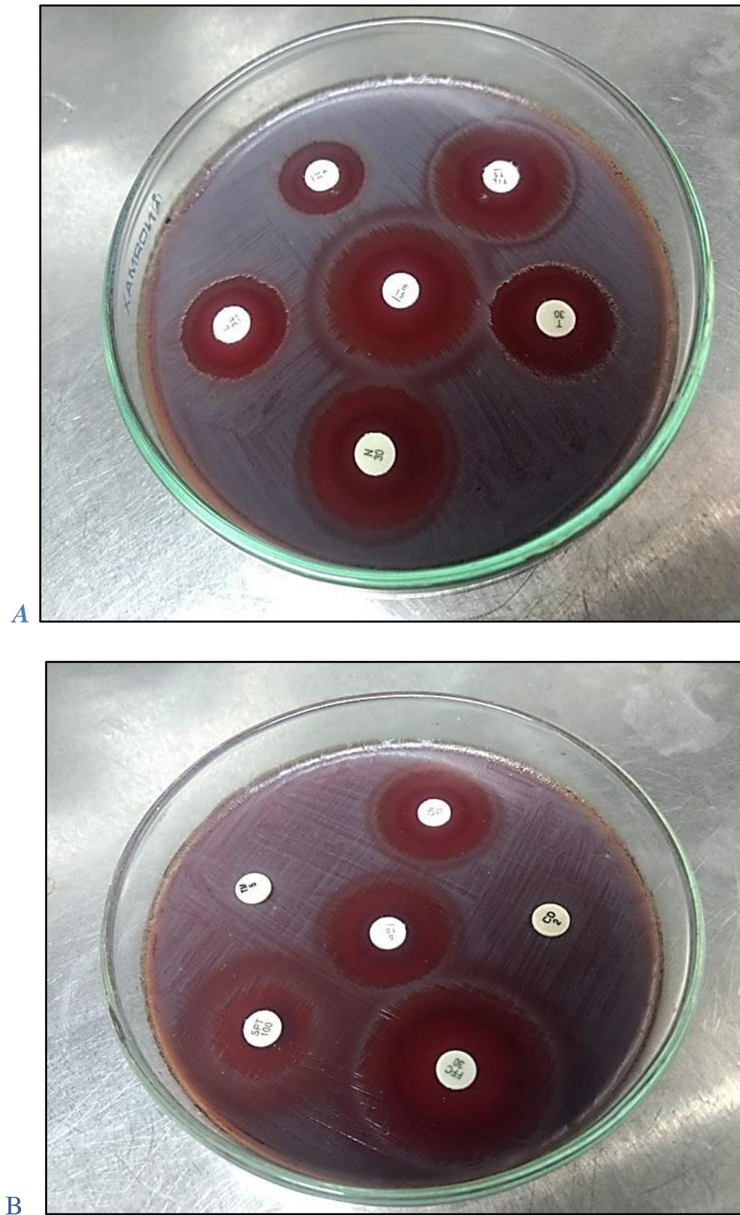

Figure 5 : (A) and (B) shows visible Zones of inhibition to all the antibiotics applied

Culturing in 96 well plate for biofilm formation: 200 µl of the diluted culture of *Pasteurella multocida* were poured in triplicate to 96-well plates. One set of plates was incubated aerobically for 96 hour at 37ºC.

Washing of 96 well plate of biofilm of *Pasteurella multocida*: On completion of incubation all the bacterial suspension from 96 well plates were removed and then the plates were washed three times with 250 µl of sterile 0.9% sodium chloride solution to remove any planktonic cells (de Emery *et al*. 2017). Then 200 µl methanol were added further to fix the adhered bacterial cells. After 15 min, the content of the wells was removed and the plates were dried at room temperature. Afterwards, 200 µl of 1% crystal violet dye were added to each well at room temperature for 5 min. Then washing of plate was done thrice in water and air dried at room temperature (Rajagopal *et al*., 2013).

Quantification of 96 well plate of Biofilm: The quantification of 96 well plates of biofilm was done by recording the absorbance at 590 nm after addition of 200

µl of 33% glacial acetic acid for 1hour at room temperature through Elisa reader. Respective media was used as control in the wells of 96 well plates.

Statistical Analysis: Data related to antibiogram assay and biofilm availability was subjected to Chi square test. The statistical analysis was performed on Statistical Package for the Social Sciences (IBM SPSS version 23) software. The statements of statistical significance were based on a probability of P<0.05.

RESULTS AND DISCUSSIONS

A total of 100 nasal and tracheal swab samples were collected from animals brought in the PAMCO (Punjab Agriculture and Meat Company) slaughter house Lahore. Samples were collected using sterile cotton swabs. All isolates grew with circular, convex, "Mousy" odor colonies on Blood Agar due to metabolic products. Smears were prepared from cultures producing moist, muciod and mousy odor colonies on Blood agar. When stained by the Giemsa staining procedure showed bipolar staining and safety pin appearance. All the isolated strains were gram negative rods as observed under microscope.

All the 76 isolates confirmed by culturing and microscopic examination were subjected to extraction of total genomic DNA. After the extraction of DNA, PCR was performed for confirmation of *Pasteurella multocida*. All 76 isolates were identified to be *Pasteurella multocida* by PCR using species specific primers set i.e. KTT72 and KTSP61. Amplified products were resolved by 2 % agarose gel electrophoresis. All isolates showed amplicons of approximately 590 bp size run at 4V/cm for 1 hour.

Antibiotics profiling was done of pure isolates on MHA (Mueller Hinton Agar) plate with 5% sheep blood. Pure isolates of *Pasteurella multocida* (n=76) were swabbed on the surface of agar plate and different antibiotics applied onto it. Zones of inhibition were observed after overnight incubation.76 isolates were subjected to test for antimicrobial resistance profile and these isolates were considered as sensitive, resistant and intermediate according to CLSI guidelines as listed. Percentage of resistant, sensitive and intermediate isolates to all antibiotics is shown in Table 1.

For the Biofilm formation, all *Pasteurella multocida* positive isolates were subjected to the culturing in 96 well plates and then stained with crystal violet. The percentage of biofilm production of all 76 isolates of *Pasteurella multocida* as shown in Table 2.

Table 1: **Antibiogram assay of 76 isolates of *Pasteurella multocida***

Antibiotics	Sensitive		Intermediate		Resistant		P-value (P<0.05)
	No. of positive samples	(%)	No. of positive samples	(%)	No. of positive samples	(%)	
Spectinomycin	18/76	23.69%	23/76	30.26%	35/76	46.05%	<0.0001
Streptomycin	15/76	19.73%	29/76	38.16%	32/76	42.11%	
Sulfadiazine	6/76	7.89%	32/76	42.11%	38/76	50.00%	
Erythromycin	3/76	3.94%	41/76	53.95%	32/76	42.11%	
Tetracycline	53/76	69.73%	8/76	10.53%	15/76	19.74%	
Neomycin	49/76	64.47%	18/76	23.68%	9/76	11.85%	
Tilmicosin	23/76	30.27%	47/76	61.84%	6/76	7.89%	
Chloramphenicol	64/76	84.21%	9/76	11.85%	3/76	3.94%	
Florfenicol	76/76	100.00%	0/76	0.00%	0/76	0.00%	
Clindamycin	0/76	0.00%	6/76	7.89%	70/76	92.11%	
Gentamycin	70/76	92.11%	3/76	3.94%	3/76	3.95%	
Kanamycin	55/76	72.37%	18/76	23.68%	3/76	3.95%	

Table 2: Distribution of biofilm assay in *Pasteurella multocida*

Type of biofilm producer	No. of Positive Samples	No. of Negative Samples	Percentage	P-value (P<0.05)
Non biofilm producer	6	70	7.89%	<0.0001
Weak biofilm producer	26	50	34.21%	
Moderate biofilm producer	35	41	46.05%	
Strong biofilm producer	9	67	11.85%	

In this study *Pasteurella multocida* was selected and considered important because it causes severe economic losses to livestock sector. So, the present study was designed to assist in the rapid detection of *Pasteurella multocida* from mixed cultures when the clinical sample is taken from a contaminated area of the animal such as the nose or throat. For isolation and molecular characterization of *Pasteurella multocida* from upper respiratory tract of apparently healthy bovines nasal and tracheal swabs were taken which were then subjected for confirmation by selective culturing on Blood agar plates and PCR were performed with the use of a specific primer sequences designed to check the prevalence of pathogen. The primers pair were used in this study produced amplification product which were unique to identify the *Pasteurella multocida* isolates. The present study also describes that PCR amplification performed directly on bacterial colonies or cultures exhibits an extremely rapid, sensitive method of *Pasteurella multocida* identification (Townsend *et al.* 2001).

Current study concluded that when 100 samples were subjected to culturing to identify the *Pasteurella multocida* colony based identification and microscopic examination then out of 100 samples, 76% (76/100) were found positive for *Pasteurella multocida* which were then confirmed by PCR based analysis. So, the present study shows that *Pasteurella multocida* is normal inhabitant and opportunistic pathogen of cattle and buffaloes. *Pasteurella multocida* develops resistance to many antimicrobial agents. The increased prevalence of antimicrobial resistant bacterial pathogens has become a major public and animal health concern. Continuous monitoring of antimicrobial susceptibility is important for the selection of potent antimicrobial agents for treatment of bovine *Hemorrhagic septicemia* (Katsuda *et al.,* 2013). Most antimicrobial agents used for the treatment of bovine Pasteurellosis are also beneficial for use in pigs, but their amount and route of administration differ more or less between cattle and pigs (Yoshimura *et al.,* 2001).

It was concluded that when all 76 pure isolates of *Pasteurella multocida* were tested for their sensitivity against different antibiotics available for the treatment of bacterial infection in animals. All of the 76 isolates were found sensitive to florfenicol (100%), gentamycin (92.11%), chloramphenicol (84.21%) and kanamycin (72.37%) respectively. For tetracycline and neomycin sensitivity were (69.73%) and (64.47%) respectively against the bacteria. Whereas tilmicosin and spectinomycin sensitivity were recorded as (30.27%) and (23.69%). Streptomycin (19.73%), sulfadiazine (7.89%), erythromycin (3.94%) and clindamycin (0.0 %) were least sensitive (Naz *et al.,* 2012).

In this study all of the pure 76 isolates of *Pasteurella multocida* were subjected to culture into micro-titer plates to see the biofilm forming ability of bacteria in in-vitro condition. In this regard many bacterial pathogens exist in animals as biofilms. Animal diseases where bacterial biofilms are formed to be involved based on histopathology and ultra-structural appearance of the bacteria within tissue. However, the biofilms increases the resistance of bacteria to host immune defense mechanisms (Olson *et al.,* 2002).

Biofilm formation capacity of *Pasteurella multocida* as it is a respiratory pathogen of many animals and biofilm mode could easily be one of its virulence factors for survival inside and outside the host (Rajagopal *et al.* 2013). The present study was undertaken to quantify biofilm formation of *Pasteurella multocida* onto 96 well micro-titer plate and measure biofilm production capability after incubation of 96 hours. After incubation of all 76 isolate, washing of the plates were done with crystal violet and optical density (OD) values recorded. It was concluded that when all pure isolates of *Pasteurella multocida* were tested for Biofilm assay out of 76 isolates categorized as: non biofilm producer (7.89%), weak biofilm producer (34.21%), moderate biofilm producer (46.05%) and strong biofilm producer (11.84%). So, it was concluded that *Pasteurella multocida* isolated from cattle and buffaloes were found to form biofilm in in-vitro condition (Prajapati *et al.* 2020)

Conclusion: In conclusion, current study gives data on the molecular characterization of *Pasteurella multocida* isolated from the healthy bovine's nasal and tracheal swab in the Lahore PAMCO slaughter house. This study also concluded that the misuse of antibiotics in livestock farming results in rise of antibiotic resistance among food producing animals which envision a major threat to production industry. So the obtained results should be considered as a possible transfer risk of this bacterium in threating the human health by the food chain.

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