

Isolation and molecular characterization of avian pathogenic *Escherichia coli* and *Pseudomonas aeruginosa* from mortality in ducks in Kashmir, India

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Received: 9 June 2009; Accepted: 18 September 2009

Key words: APEC, *Escherichia coli*, Exotoxin A iss, *Pseudomonas aeruginosa*

A group of *Escherichia coli* (*E. coli*) collectively termed as avian pathogenic *E. coli* (APEC) causes colibacillosis in poultry and other avian species. APECs most likely enter and colonize the avian respiratory tract by inhalation of faecal dust, leading to localized infections such as septicaemia, cellulitis, air sacculitis and pneumonia (Dho-Moulin and Fairbrother 1999). A variety of virulence attributes of APEC that include increased serum survival (*iss*), presence of type 1 fimbriae (*fimC*), temperature sensitive haemagglutinin (*tsh*), haemolysin (*hlyE*), S-fimbriae (*sfa*) and colicin V (*cvaC*) are implicated in promoting these extraintestinal diseases in avian species (McPeake *et al.* 2005).

Pseudomonas aeruginosa (*Ps. aeruginosa*) a gram negative bacterium is implicated in various infections including lung infections in man and animals. *Ps. aeruginosa* produces an extracellular toxin called exotoxin A, which is encoded by *exoA* gene (Khan and Cerniglia 1994) and is 10 000 times more toxic to mice than *Pseudomonas* endotoxin (Iglewski and Kabat 1975). Present work describes the isolation and molecular characterization of APEC and *Ps. aeruginosa* associated with heavy mortality in ducks in an organised farm in Kashmir valley.

An outbreak of disease occurred in ducks (Khaki Campbell layer type) at Duck Farm, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-K, Shuhama (Alusteng), Srinagar, in December 2007. The ducks were aged between 6 and 10 weeks. Out of 250 ducks, 199 died during the outbreak. The affected ducks showed paralytic signs and were unable to stand. Some of the ducks exhibited lameness. Most of the ducks suffered from greenish watery

diarrhoea. Necropsy revealed echymotic and petechial haemorrhages on heart and liver, respectively. Besides, spleen and kidneys were swollen.

Isolation of bacteria: Heart blood, pieces of liver, lung and intestine of 6 duck carcasses referred to this Division were plated onto nutrient agar, blood agar and MacConkey agar, and incubated aerobically at 37°C for 24 h. The well-separated colonies were picked up onto nutrient agar slants as pure culture and subjected to standard morphological, biochemical tests (Holt *et al.* 1994) to ascertain their identity. The isolates were identified as *E. coli* and *Ps. aeruginosa*.

Screening for presence of virus: Besides, morbid material was inoculated into Vero cell monolayer to look for presence of virus permissible to Vero cells. The liver and lung tissues of dead ducks were homogenised with homogenizer and centrifuged at 10 000 × g. Supernatant was filtered through 0.2µm syringe filter and used for inoculation on vero cell monolayer for detection and isolation of virus. A vero cell line was grown in a 12-well cell culture plate in a CO₂ incubator at 37°C under 5% CO₂ tension and 200 µl of tissue filtrate was inoculated into duplicate wells. Additional two wells without any inoculums served as negative control. The plate was incubated as before. Cells were examined for cytopathic effects (CPE) after every 24 h up to 72 h using an inverted microscope.

Screening of *iss*, *sfa*, *cvaC*, *tsh*, *hlyE* and *fimC* virulence genes of APEC and *exoA* gene of *Ps. aeruginosa*: For detection of various virulence specific genes bacterial DNA was extracted (Wani *et al.* 2006). The *iss*, *sfa*, *cvaC*, *fimC*, *hlyE* and *tsh* genes of APEC were detected as per McPeake *et al.* (2005). APEC strain belonging to serogroup O12 was used as positive control for *iss* gene. The *exoA* gene of *Ps. aeruginosa* was detected as per the protocol and primers described by Xu *et al.* (2004). Amplified PCR products were analysed by gel electrophoresis in 1.5–2% agarose gel containing ethidium bromide (0.5µg/ml). The products were visualised with UV illumination.

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Vero cytotoxicity assay: The *Ps. aeruginosa* was inoculated into 5 ml of Luria-Bertani (LB) broth and incubated overnight at 37°C in the shaking incubator. The overnight culture was centrifuged at $10\,000 \times g$. Cell free supernatant was filtered through 0.2µm syringe filter and used for cytotoxic assay. Sixty microlitre of cell free filtrate (CFF) was inoculated into duplicate wells of vero cell monolayer and incubated as described above. Equal volume of sterile LB broth instead of CFF served as negative control. The plate was incubated and observed for CPE as before.

Serogrouping of APEC was carried from National *Salmonella* and *Escherichia* centre, C.R.I., Kasauli (Himachal Pradesh).

In vitro antibiotic susceptibility testing: The *in vitro* antibiotic sensitivity tests of the *E. coli* and *Ps. aeruginosa* isolates were carried out by disc diffusion technique as described by Bauer *et al.* (1966).

Present study records the investigation of heavy mortality in an organised duck farm in Kashmir valley. Two types of organisms were isolated from all the 6 samples subjected to bacteriological examination. One isolate was identified as APEC and the another one as *Ps. aeruginosa*. The APEC isolate was untypeable (UT) and possessed *iss* gene as it produced an amplicon of 323 bp (Fig.1) by PCR but did not harbour *sfa*, *cvaC*, *tsh*, *hlyE* or *fimC* virulence genes. There seems to be no report available about the isolation of APEC with *iss* gene from ducks. However, there are reports of the isolation of APEC strains harbouring *iss* gene from outbreaks of heavy mortality in poultry (McPeake *et al.* 2005, Rodriguez-Siek *et al.* 2005). The *iss* gene was first identified in a human septicaemic *E. coli* isolate (Binns *et al.* 1982)

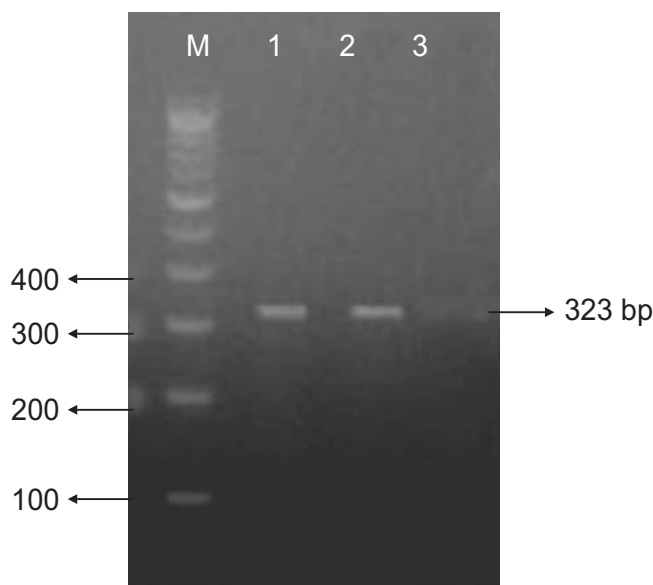


Fig 1. Detection of *iss* gene in APEC isolate by PCR. Lane M: 100 bp DNA ladder, Lane 1: APEC isolate positive for *iss* gene, Lane 2: positive control, Lane 3 negative control.

and was associated with a 20-fold increase in complement resistance and a 100-fold increase in virulence toward 1-day-old chicks (Chuba *et al.* 1989). An association between pathogenicity and serum resistance has been previously demonstrated by Knobl *et al.* (2001). The *iss* gene was also demonstrated among the isolates from cellulitis and colisepticaemia in chickens (Jeffrey *et al.* 2002). Tivendale *et al.* (2003) detected the *iss* gene in 3 highly virulent strains of APEC, and suggested that it is required in association with *iucA* (aerobactin operon) gene for the highest level of virulence.

The *Ps. aeruginosa* isolate revealed the presence of *exoA* gene as it yielded a product of 396 bp (Fig.2) by PCR. It is a specific marker of *Ps. aeruginosa* (Xu *et al.* 2004), which codes for exotoxin A. The role of exotoxin A in the mortality of experimentally infected mice has been demonstrated (Manafi *et al.* 2009). Woods *et al.* (1982) assessed the role of *Ps. aeruginosa* *exoA* in chronic lung infection in the rat model, and demonstrated that active toxin A is required for maximum virulence. They observed that chronic inflammatory changes could be induced only with toxigenic strain. It appears that the virulence potential of exotoxin A may vary with the site and tissue involved (Xu *et al.* 2004). Cell free filtrate of *Ps. aeruginosa* produced prominent CPE on vero cells after 48 h. No CPE was observed on vero cell monolayer, inoculated with tissue filtrate, even after 72 h of inoculation. The CPE on vero cells due to CFF of *Ps. aeruginosa* may be due to its exotoxin A. However, this needs to be investigated further. Absence of CPE in vero cells after inoculation of filtered tissue homogenate was suggestive of absence of virus permissible to vero cells.

Determination of *in vitro* antimicrobial susceptibility of the bacterial isolates is prerequisite for judicious use of

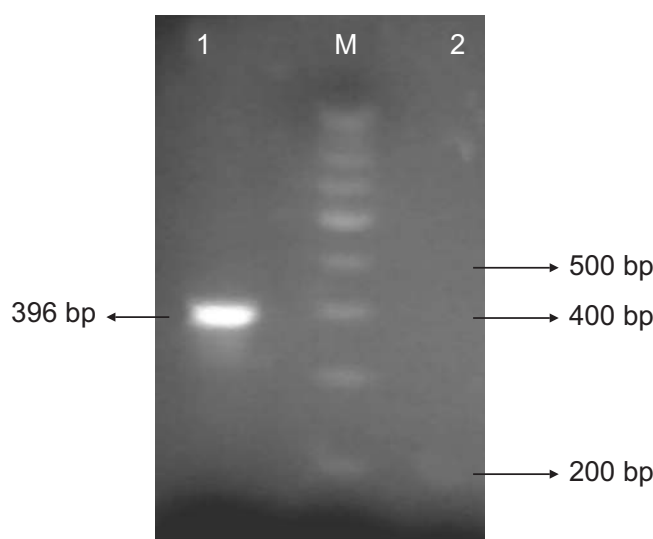


Fig 2. Detection of *exoA* gene in *Pseudomonas aeruginosa* by PCR: Lane 1: *Pseudomonas aeruginosa* isolate positive for *exoA* gene. Lane M: 100 bp DNA ladder Lane 2: negative control.

antimicrobials for effective treatment and to avoid the development of drug resistance. The APEC isolate was sensitive to gentamicin and ampicillin/cloxacillin while *Ps. aeruginosa* was sensitive to gentamicin, ciprofloxacin, enrofloxacin and ampicillin/cloxacillin. These are the antibiotics less abused in poultry industry. Both isolates were resistant to tetracycline, norfloxacin, cephalixin, nalidixic acid and doxycycline. In addition the APEC was also resistant to ciprofloxacin and enrofloxacin. This multi drug resistance of the isolates might be due to indiscriminate use of antibiotics in clinical practice. These resistance levels are comparable to those previously reported for APEC by other investigators (Saha *et al.* 2007) and for *Ps. aeruginosa* by Sekiguchi *et al.* (2007). Antibiotic therapy with gentamicin and ampicillin/cloxacillin checked the mortality of ducks at the farm.

Keeping in view the symptoms and lesions of the affected birds and the virulence attributes of the isolates, it appears that APEC and *Ps. aeruginosa* isolates could have been the cause of heavy mortality in ducks.

SUMMARY

Present study records the investigation of heavy mortality in an organised duck farm in Kashmir valley. Bacteriological examination of the morbid material revealed the presence of avian pathogenic *Escherichia coli* (APEC) and *Pseudomonas aeruginosa* (*Ps. aeruginosa*). The APEC isolate carried *iss* virulence gene but lacked *sfa*, *cvaC*, *tsh*, *hlyE* or *fimC* virulence genes. The *Ps. aeruginosa* revealed the presence of *exoA* virulence gene. The cell free filtrate of *Ps. aeruginosa* produced prominent cytopathic effect (CPE) on vero cells after 48 h. The tissue filtrate from morbid material did not produce CPE on Vero cells. Both the isolates were resistant to tetracycline, norfloxacin, cephalixin, nalidixic acid and doxycycline but sensitive to gentamicin and ampicillin/cloxacillin. Treatment of the affected flock with gentamicin and ampicillin/cloxacillin was effective to check the mortality at duck farm. This appears to be first report of its kind from India.

ACKNOWLEDGEMENT

The authors are grateful to Professor Anwar Alam, Vice-Chancellor, SKUAST-Kashmir, for providing facilities. Thanks to Director, National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli 173204 (Himachal Pradesh) for serogrouping of the APEC isolate. The Vero cell line used in this study was kindly supplied by Dr T N Naik, NICED, Belighata, Kolkata 700 017

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