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2 Experimentally infection of Cattle with wild types of Peste-des-petits- 3 ruminants Virus – Role in its maintenance and spread.

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5 Running title : Infection of Cattle with PPRV

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which can infect also cattle without any clinical signs but show a seroconversion. However the epidemiological role of cattle in the maintenance and spread of the disease is not known. For the purpose of the present study, cattle were infected with a wild candidate from each of the four lineages of PPRV and placed in separate boxes. Then naive goats were introduced in each specific box for the 30 days duration of the experiment. The results showed that no clinical signs of PPR were recorded from these infected cattle along with the in-contact goats. The nasal and oral swabs remainend negative. However, animals infected with wild types of PPRV from lineages 1, 3, 4 seroconverted with high percentage inhibition (PI % = values.

27 Only two animals out of three with the Nigeria 75/3 strain of lineage 2 (mild strain) did elicit
28 a production of specific anti-PPR antibodies in those cattle but with PI% values around the
29 threshold of the test. Our findings confirm that cattle are dead end hosts for PPRV and do not
30 play an epidemiological role in the maintenance and spread of PPRV. In a PPR surveillance
31 programme, cattle can serve as indicators of PPRV infection.

32 Key words : Cattle , small ruminant, PPR, Morbillivirus,

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34 Importance

35 Peste-ds-ptetis-ruminants (PPR) is a major Transboundary Animal disease (TADs) in the
36 tropical regions which is spreading extensively nowadays to southern and northern of Africa,
37 Turkey in Europ and southwest Asia. PPR virus is very close related to Rinderpest virus
38 (RPV) which has been eradicated from the world . Today FAO, WOAH / OIE and the
39 scientific community have elected PPR to be the second animal disease to be eradicated
40 through The PPR Global Eradication Programme (GEP-PPR). Since PPR infects cattle
41 without any clinical signs but they seroconvert, it is important to explore the role of cattle in
42 the maintenance and spread of PPRV to better understand the epidemiology of the disease
43 which will help in the the GEP-PPR.

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45 Introduction

46 Peste Des Petits Ruminants (PPR) is a serious and contagious plague of small ruminants,
47 mostly sheep and goats, in many developing countries in Africa, near and Middle-East and
48 southern Asia (1, 2)). Within Africa, PPR has now extended to southwards in Tanzania,
49 Democratic Republic of Congo and Angola (3, 4). Outbreaks of PPR have been also reported
50 across North Africa including Algeria, Morocco, Tunisia (5, 6) along with the European part
51 of Turkey (7). In southwest Asia, China has reported PPR spread all over the country starting
52 during year 2007 in Tibet region (8). The current spread of PPR over large geographical areas
53 is certainly a result of intensified animal movement and trade but may also be due to the
54 eradication of RPV that affected small ruminants and induced immunity against PPR. Animal
55 of all ages are susceptible and the transmission route remains oral and respiratory secretions
56 following close contact between infected and naive population (9).

57 The causative agent, Peste Des Petits Ruminants Virus (PPRV) is a negative-stranded RNA
58 virus with a monosegmented genome of length 15,948 and containing six genes encoding six
59 structural proteins. It belongs to family *Paramyxoviridae* and the genus *Morbillivirus* together
60 with Rinderpest Virus (RPV), Measles Virus (MV), Canine Distemper Virus (CDV) and
61 marine mammalian Morbilliviruses (10, 11). There are four lineages of PPRV based on the
62 differentiation determined by the sequence comparison of a small region of the F gene (12) or
63 the N gene (13). However, it has been demonstrated recently that the N gene is more
64 divergent therefore more suitable for phylogenetic distinction between closely related PPRV
65 viruses (14).

66 The disease is highly contagious and case fatality rates in some outbreaks can approach 90%
67 in susceptible populations and, as a consequence of the effects of epidemics, the local and
68 rural economies of the affected countries can be devastating (15, 16). Nowadays there are
69 efficient attenuated vaccines to be used to prevent this disease and to control its extension (17,
70 18).

71 PPRV infects also cattle but only causes disease in small ruminant species while a specific
72 seroconversion to PPR is observed in cattle (19). However, a high mortality of domestic
73 buffaloes (*Bubalus bubalis*) was noted in India caused by an infection with PPRV (20). Even
74 though this situation has not been reported again, there is a necessity to clarify it
75 experimentally and by collection of data from rural communities where mixed species (cattle
76 and small ruminants) graze together.

77 The present study aimed to investigate the epidemiological role of cattle in the maintenance
78 and spread of PPRV among cattle and small ruminants‘populations.

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87 Material and Methods

88 Animal

89 Cattle : 15 individuals (N'dama breed), two-three years old, were randomly selected from a
90 farm belonging to the Centre for Research in Agronomy (CNRA – La Mé), located at
91 approximatively, 30 kms from Abidjan. They were tested as being negative for antibodies to
92 PPRV using a competitve ELISA (21). Then they were housed in boxes with separate feeding
93 and drinking tanks. These animals were treated with the anthelmintic Albendazole (10mg/kg)
94 two times during the acclimatisation period lasting ten days.

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96 Goats : 15 West African dwarf goats, randomly selected from the same centre (including
97 seven control goats), aged one - two years, which were tested negative for the presence of
98 antibodies against PPR by PPR competitive ELISA (c-ELISA) (21), were used for the study.
99 Each animal was treated with the anthelmintic Albendazole (7.5 mg/kg) two times during the
100 acclimatisation period (including infected control and uninfected control goats).

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102 After 10 days for the acclimatisation period, the 15 individuals cattle were, at random, divided
103 in four groups of three each with the fifth group (conrol) having also three animals. Each
104 group was randomly assigned to one specific box corresponding to a specific PPRV lineage
105 (Table1).

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107 All animals in the experiment were earmarked with a unique identification number.

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109 Virulent isolates used in challenge

110 Four virus isolates were obtained from the virus bank of CIRAD-Montpellier (France)
111 representing viruses from different geographical regions and belonging to different lineages
112 based on the sequences of their nucleoprotein (NP) gene (14, 22): CIV89 (Lineage 1),
113 Nigeria 75/3 (lineage 2), Ethiopia (lineage 3), India-Calcutta (lineage 4).

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117 Virulent challenge

118 Each individual cattle (except uninfected controls) was infected subcutaneously with 1 mL of
119 the various challenge viral suspensions, at a concentration of 10^3 TCID₅₀/mL. Animals were
120 kept separately in boxes. Three cattle were not infected and used as controls.

121

122 Infected Control goats : two goats were infected subcutaneously with 1 mL of CIV89 strain,
123 at a concentration of 10^3 TCID₅₀/mL and two goats infected with India-Calcutta strain at the
124 same concentration.

125

126 Uninfected control goats : three goats were not infected.

127

128 Twenty four hours (24h) after the virulent challenge of cattle, randomly two uninfected and
129 naive goats were introduced into each box already containing infected cattle with a sepecific
130 challenge strain of PPRV.

131 Infected goats with CIV89 and with India-Calcutta strains respectively, were kept in separated
132 boxes in another animal building. Uninfected control goats were kept in a different box in the
133 same building.

134

135 An attendant was assigned to each box to feed and water the infected and control animals.

136 Animals were examined daily for classical signs of PPR and body temperatures were recorded
137 for first ten days post infection (pi) then only for clinical examination up to 30 days pi for
138 cattle.

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140 The study was approved by the Ethics Committee of LANADA – Abidjan and by the National
141 Ethics Committee - Ivory-Coast. In addition the principal investigator and corresponding
142 author was certified from the **International Council for Laboratory Animal Science**
143 (ICLAS).

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150 Sample collection

151 Serial bleeding was performed on all animals at : day0, day2, day5, day7, day9 then day15,
152 day30 post infection (end of the study) for cattle and in-contact goats and up to day8 for
153 infected control goats. Serum was separated and samples stored at -20°C until examined.

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155 Swab : ocular and nasal swabs were collected at day0, day2, day5, day7, day9 then day15,
156 day21 and day30 post infection, for cattle and in-contact goats. Individual sterile swabs were
157 used in the present experiment. In the Centre, collected swab samples were kept in liquid
158 nitrogen to prevent any degradation of biological materials. At the laboratory, swabs were
159 transferred to a -80°C freezer until used for analysis.

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161 Serological test

162 A competitive ELISA (cELISA) kit (CIRAD-Montpellier, France), based on a recombinant
163 NP was used to detect specific antibodies against PPR (21) following recommended
164 protocols. Fifty microlitres were used throughout. Maxisorp 96-wells plates were coated with
165 the recombinant NP antigen diluted 1/1600 in PBS (0.01 M, pH 7.2–7.4) and incubated at
166 37°C for 1 h on an orbital shaker. After a cycle of three washes in phosphate buffered saline
167 (PBS; 1/5, 0.05% Tween 20), test serum (5 µL), was added to 45µL of blocking buffer (PBS
168 0.01 M. pH 7.2–7.4; 0.05% Tween 20 (v/v); 0.5% negative sheep serum (v/v)) followed
169 immediately by the addition of 50µL of the specific monoclonal antibody (Mab) against the
170 PPRV NP at a dilution of 1/100 in blocking buffer. Control sera included were, strong
171 positive, weak positive, negative and a Mab control (0% competition). The plates were
172 incubated and washed as above. Anti-mouse horse radish peroxidase enzyme conjugate
173 (DAKO A/S), diluted 1/1000 in blocking buffer, was added and plates incubated as before.
174 The plates were washed and 50µL of substrate/ chromogen (H₂O₂/OPD) were added and the
175 colour allowed to develop for 10 min, after which time any reaction was stopped by the
176 addition of 50µL of sulphuric acid (1 M.). Plates were read on an ELISA reader (Multiskan
177 MK II) at an absorbance of 492 nm. Optical density (OD) readings were converted to
178 percentage inhibition (PI) values using the following formula:

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180 PI% = 100 (OD in test well / OD in 0% control well) x100.

181 PI% values greater than or equal to 50% were considered positive

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184 Single stranded cDNA synthesis and PCR technique

185 Oral and nasal swabs were processed as described (13). The procedure for RNA isolation was
186 as recommended by the manufacturer, using the RNeasy Mini Kit (Qiagen, Germany). The
187 RNA was eluted in 50 µL of nuclease-free water. The RT step was performed by using

188 random hexamer primers (Introgen, Carlsbad, CA., USA) with 10 µL of extracted RNA and
189 the First-strand cDNA Synthesis Kit (GE Healthcare Europe GmbH, Orsay, France) as
190 recommended by the manufacturer's protocol. Then, 5 µL of the cDNA obtained was used as
191 the template for the PCR step in a 200 µL thin wall tube. The PCR was carried out using the
192 Gene Amp PCR system 2400 (Perkin-Elmer, Applied Biosystems, Paris, France) using a 50
193 µL reaction mixture with the specific set of primers NP3 (forward: 5' – TCT CGG AAA TCG
194 CCT CAC AGA CTG) and NP4 (reverse:
195 5' – CCT CCT CCT GGT CCT CCA GAA TCT) as previously outlined (13) targeting a
196 fragment of 350 bp on the nucleoprotein (NP) with the following programme: an initial
197 denaturation step at 95°C for 5 min followed by five cycles with denaturation at 94°C for 30
198 sec, annealing at 60°C for 30 sec and the extension at 72°C for 30 sec. Then the amplification
199 process continued for 30 cycles more but in which the annealing temperature was reduced to
200 55°C. The amplification reaction was terminated by a final extension of 10 min at 72°C.
201 Negative and positive controls were included in all experiments.

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207 Results

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209 Clinical response of goats (Infected Control Goats) to infection with PPRV
210 CIV 89 and India-Calcutta isolates

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212 For both PPRV strains used, the infected goats developed pyrexia after an incubation period
213 of 2–7 days, with rectal temperatures ranging from 39 to 41°C. Ocular and nasal discharges
214 developed at day 4 with CIV89 strain and at day 7 post-infection with India-Calcutta strain.
215 Oral ulceration and necrotic lesions appeared at day 5 with CIV89 strain and at day 8 with
216 India-Calcutta. Diarrhoea was recorded in all infected goats. At day 8, all infected goats were
217 humanely slaughtered and samples were taken on autopsy for analysis.

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219 Uninfected goats (Control goats): No clinical signs were recorded in these control animals
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222 Clinical response of Cattle infected with isolates of PPRV from each of the four lineages
223
224 The PPRV isolates, CIV89, Nigeria 75/3, Ethiopia, India-Calcutta, representing the PPRV
225 four lineages were used to infect young cattle (three animals / PPRV strain lineage).
226 Rectal temperature remained stable between 38 and 39°C during the observation period. Only
227 one animal in the CIV89 group reached 39.7°C for 3 days. No clinical signs were recorded
228 during the whole observation period.
229
230 In-contact goats: No clinical signs were observed in these animals.
231 Control Cattle: No clinical signs were recorded in these control animals as well.
232
233 Serological response of goats to infection with PPRV isolates
234 The four infected goats with CIV89 and India-Calcutta respectively seroconverted at day7 and
235 the uninfected controls remained sero negative.
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241 Serological response of Cattle to infection with PPRV isolates
242 All the infected cattle with PPRV isolates were negative from day0 to day7 post infection
243 after analysis of the respective serum samples with the cELISA technique. At day9, 6/12
244 became positive, 11/12 positive at day15 and 11/12 positive at day30. One animal of group2
245 (cattle infected with Nigeria 75/3, lineage2) did not seroconvert. The PI values of the positive
246 individuals in this group 2 ranged between 50 and 54% while these values were above 65%
247 for positive animals in groups 1, 3 and 4.
248 The control animals remained negative (Table2).
249
250 All in-contact goats introduced in each specific box containing infected cattle with each
251 specific lineage of PPRV remained negative.
252
253 Detection of viral genome

254 All swab samples (ocular and nasal swabs) from infected cattle with PPRV and in-contact
255 goats were analysed using the PCR technique on cDNA generated with random hexamers.
256 This analysis found that all collected swabs were negative along with those taken from control
257 animals (Table2).

258 Samples collected from slaughtered goats (infected controls) were positive by amplifying the
259 targeted fragment of 350 bp of the NP gene.

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261 Discussion

262 PPR is a dreadful disease of sheep and goat being a real burden on the development of these
263 species with goat being affected more severely than sheep (15). Within goat species, there is
264 a difference in the susceptibility to PPRV between sahelian long-legged goat breed and West
265 African dwarf goat breed from the tropical forest region with the latest more susceptible (23,
266 24). Conversely, PPRV is not considered as pathogenic in cattle, domestic, and wild African
267 buffaloes (*Syncerus caffer*) (25) while they can seroconvert after infection with PPRV (7, 26,
268 27). However, high case fatality rates (96%) were reported in India in domestic buffaloes
269 (*Bubalus bubalis*) and the disease was experimentally reproduced in these animals (20, 25). In
270 Ivory-Coast, a survey on wildlife in the National game park of Comoé during the Global
271 Rinderpest Eradication Programme (GREP) revealed that 1/56 serum samples and three pools
272 of five swabs samples each collected from African wild buffaloes (*Syncerus caffer*) were
273 positive to PPRV (28). This national park harbors some villages having domestic sheep and
274 goats and contacts with wild ruminants are frequent which contribute to cross-species
275 transmission of PPRV.

276 No other cases have been reported from India since then or elsewhere in Africa in cattle or
277 African buffaloes populations. Our study was designed to give an answer to the infection of
278 cattle with PPRV and to demonstrate whether cattle can play an epidemiological role in the
279 spread of PPRV infection among cattle and small ruminants' populations. Previous study
280 implemented in Africa with PPR virus strains from each lineage demonstrated that CIV 89
281 (Lineage 1) strain is highly virulent followed by India-Calcutta (Lineage4 then Ethiopia
282 (Lineage3) and finally Nigeria 75/3 strains (Lineage2) (24). In our study, control goats
283 challenged with CIV89 and India-Calcutta strains developed clinical signs consistent with
284 PPR and were humanely sacrificed at day8 post infection, which confirmed the virulence of
285 PPR virus strains used in this experiment. In addition, laboratory analysis on samples

286 collected from these animals confirmed the disease. Infection of cattle with PPRV strains
287 from each lineage did not show any clinical signs during the observation period of 30 days
288 along with the in-contact naive goats introduced in the respective boxes like the control cattle
289 and non-challenged control goats. This result demonstrated that cattle, after infection with
290 PPRV, there is no replication, at least at the level of the epithelial cells (no investigation of the
291 others cells such as PBMC) and do not excrete the virus able to contaminate animals in close
292 contact such as goats placed in the same box. The absence of viral excretion from these
293 challenged cattle is confirmed by the negative results of the collected swabs using the RT-
294 PCR technique. Furthermore, recently, authors carried out an experimentally infection of
295 calves with PPRV and could demonstrate the presence of PPRV antigen and nucleic acid in
296 blood, plasma and PBMCs during a long period. They concluded that cattle pose no risk in
297 transmitting the disease as virus was absent of the natural secretion of the animals (29).

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299 Analysis of the serum samples revealed a serconversion from day9 post-infection with 6
300 positive cattle out of 12, in group1 with CIV89, group3 with Ethiopia and group4 with India-
301 Calcutta strains respectively. At day15, all animals in these groups 1, 3 and 4 became positive
302 (9/12) and at day30 post-infection, these animals remained positive. However, only 2/3
303 animals , challenged cattle in group2 with Nigeria 75/3 did seroconvert. The in-contact goats
304 remained seronegative. Our study showed that, even though there is no viral excretion, the
305 challenged animals could elicit specific anti-PPR antibodies.

306 These findings from the infected cattle confirmed previous studies where cattle developed
307 specific humoral response and the production of antibodies to naturally or experimentally
308 infection with PPRV (10, 29-33) or with the PPR vaccine (25, 34). Furthermore, these data
309 confirm what is observed in rural communities where small ruminants and cattle co-exist,
310 grazing together on the same pasture. In consequence, cross-species transmission of PPRV
311 from small-ruminants to cattle is likely to occur frequently (4). At day7 post-infection, none
312 of cattle responded serologically to the challenge with PPRV while sheep and goats
313 seroconvert earlier, at day7 post infection or after vaccination (24). The weak seroconversion
314 of animals in group2 with Nigeria 75/3 strain (2/3 positive animals with PI values just above
315 the threshold) seems to be likely linked to the virulence of the strain of PPRV. Indeed,
316 challenged animals with strains from lineages 1, 3 and 4 induced a correct production of
317 specific antibodies against PPRV. A study revealed that challenged goats with this PPRV

318 strain 75/3 survived after showing mild to inapparent PPR disease and seroconverted (24).
319 The present results from group2 confirm previous study where 66 animals seroconverted out
320 of 93 (71%) young cattle vaccinated with the PPR vaccine 75/1. A second vaccination was
321 carried out on the 27 negative animals (93-66) to obtain 100% positive animals (34).

322 We have demonstrated that cattle challenged with wild-type PPRV from each lineage do not
323 excrete the virus in the environment to contaminate in-contact animals. However these
324 animals seroconvert following a challenge with virulent wild-types PPRV. Therefore cattle
325 cannot be considered as a PPRV reservoir and do not play an epidemiological role in the
326 maintenance and spread of PPRV among cattle and small ruminant's populations. Cattle are
327 regarded as dead end host for PPRV and can rather serve as indicators of PPRV circulation
328 and useful animal population for surveillance in the contexte of PPR eradication programme.
329 The results of this study are of importance to be taken into account in the current PPR global
330 eradication programme.

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Table1 : Infection of Cattle with each wild type candidate from the four PPRV lineages

	Group1-Box1	Group2-Box2	Group3 - Box3	Group4 - Box4	Group5-Box5
PPRV strains	Lineage1 : CIV89	Lineage2 : Nigeria 75/3	Lineage3 : Ethiopia	Lineage4 : India-Calcutta	Control Cattle
Species					
Cattle	732	743	752	695	780
	761	764	772	741	781
	772	782	776	767	785
24h after infection : Introduction of naive in-contact goats					
In-contact	1.1	2.1	3.1	4.1	
Goats	1.2	2.2	3.2	4.2	
Separated building					
Infected	Box1 :		Box2 :		
Control goats	CCIV1*		Clnd1**		
	CCIV2		Clnd2		
Control Naïve	CN1***, CN2, CN3 : Uninfected control goats in box 4				
goats					

(*) : Control goat infected with CIV89.

(**) : Control goatinfected with India Calcutta.

(***) : Control naive goat : no challenge with any PPRV strain.

Table2 : PPR specific antibodies and genome detection Results after infection of cattle with wild type of PPRV

Lineage	Animal Identification	cELISA			RT-PCR
		Day9 pi	Day15 pi	Day30 pi	
	732	+	+	+	
1	761	+	+	+	Neg
	771	+	+	+	
	743		+	+	
2	764	Neg	-	-	Neg
	782		+	+	
	752	-	+	+	
3	772	+	+	+	Neg
	776	+	+	+	
	695	-	+	+	
4	741	-	+	+	Neg
	767	+	+	+	
Control cattle	780				
	781	Neg	Neg	Neg	Neg
	785				
In-contact goats	1.1 – 1.2				
	2.1 – 2.2	Neg	Neg	Neg	Neg
	3.1 – 3.2				
	4.1 – 4.2				
Infected control goats	CCIV1 – CCIV2		Positive at day7pi		Positive
	CIND1 – Cind2		Slaughtered at day8 pi .		Positive
Control goats	CN1-CN2-CN3	Neg	Neg	Neg	Neg

Neg : Negative